



TITLE:

# Mechanistic Studies of Alanine Racemase from *Bacillus stearothermophilus*( Dissertation\_全文)

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**Mechanistic Studies of Alanine Racemase from**  
***Bacillus stearothermophilus***

**Akira Watanabe**

**1999**

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## ABBREVIATIONS

AspAT	L-aspartate aminotransferase
BCAT	branched chain L-amino acid aminotransferase
Bis-tris propane	1,3-bis[tris(hydroxymethyl)-methylamino]propane
BSAR	alanine racemase from <i>Bacillus stearothermophilus</i>
CD	circular dichroism
CAPS	3-(cyclohexylamino) propanesulfonic acid
CHES	2-(cyclohexylamino) ethanesulfonic acid
D-AAT	D-amino acid aminotransferase
DEAE	diethylaminoethyl
dpm	disintegration per minute
$F_o$	observed structure factor
$F_c$	calculated structure factor
HEPES	[2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid
HPLC	high performance liquid chromatography
L-Ala-P	( <i>R</i> )-1-aminoethylposphonic acid
LDH	L-lactate dehydrogenase
NADH	$\alpha$ -nicotinamide adenine dinucleotide, reduced form
NMR	nuclear magnetic resonance
PEG	poly(ethylene glycol)
PLP	pyridoxal 5'-phosphate

PLP-L-Ala	<i>N</i> -(5'-phosphopyridoxyl)-L-alanine
PLP-D-Ala	<i>N</i> -(5'-phosphopyridoxyl)-D-alanine
PMP	pyridoxamine 5'-phosphate
rms	root mean square
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TLC	thin-layer chromatography
Tris	tris(hydroxymethyl)aminomethane



# Chapter 1

## General Introduction

Alanine racemase [EC 5.1.1.1] depending on pyridoxal 5'-phosphate (PLP) occurs widely in bacteria and plays a central role in the metabolism of D-alanine, an essential component of the peptidoglycans in bacterial cell walls (1).

The generally accepted mechanism of alanine racemase reaction is shown in Scheme 1-1. PLP bound with the active-site lysyl residue (A) reacts with a substrate to form an external Schiff base (B) through transaldimination. The subsequent  $\alpha$ -hydrogen abstraction results in the formation of a resonance-stable deprotonated intermediate (C). If reprotonation occurs at the  $\alpha$ -carbon of the substrate moiety on the opposite face of the planar intermediate (C), then an antipodal aldimine (D) is formed. The  $\epsilon$ -amino group of the lysyl residue is substituted for the isomerized amino acid through transaldimination, and the internal aldimine (A) is regenerated. In  $\alpha$ -hydrogen abstraction, two mechanisms have been proposed; "one-base mechanism" and "two-base mechanism". In one-base mechanism, a single catalytic amino acid residue abstracts  $\alpha$ -hydrogen from substrate and returns a hydrogen to the deprotonated intermediate (2).

In two-base mechanism, two different bases participate in the catalysis; one abstracts the hydrogen from a substrate and the other returns a hydrogen to the deprotonated intermediate (3).

Recently, kinetic analyses (4, 5) and x-ray crystallographic studies (6-8) have suggested that the alanine racemase reaction proceeds through a two-base mechanism. One catalytic residue serving as a hydrogen acceptor and the other serving as a hydrogen donor are situated on the opposite sides of the plane of internal Schiff base. In this work, I have studied the detailed reaction mechanism of alanine racemase of *Bacillus stearothermophilus* with focusing on the catalytic residues.

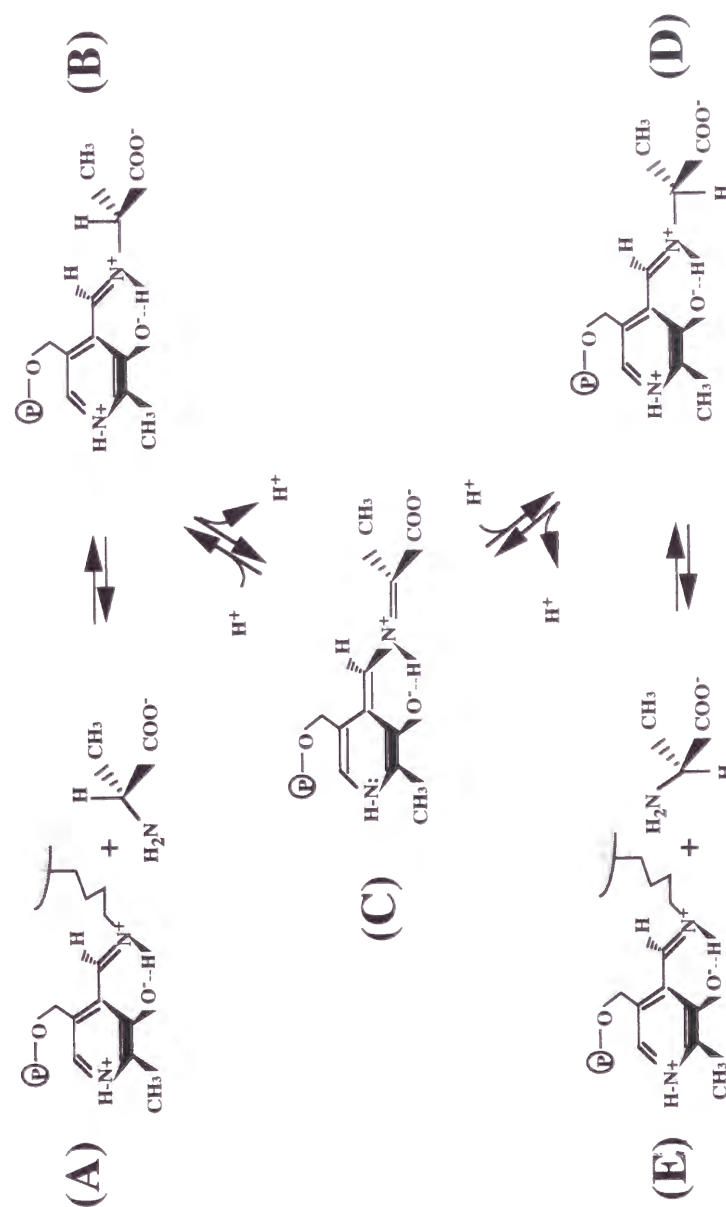
In Chapter 2, I describe the role of lysine39 (Lys39) anchoring PLP. Through the chemical rescue studies of the Lys39→Ala (K39A) mutant alanine racemase by alkylamines, I have concluded that Lys39 abstracts  $\alpha$ -hydrogen from D-alanine and transfers a proton from water to the  $\alpha$ -position of the deprotonated intermediate to form D-alanine. The obtained results also suggest that another residue serves as a catalyst specific to L-alanine.

In Chapter 3, I show the evidence that tyrosine265' (Tyr265') is the catalytic residue abstracting  $\alpha$ -hydrogen specifically from L-alanine and returning proton to the  $\alpha$ -position of the deprotonated intermediate to form L-alanine. Alanine racemase of *B. stearothermophilus* catalyzes the transamination as a side-reaction:

the enzyme transfers the amino group from both D- and L-alanine to PLP to produce pyruvate and pyridoxamine 5'-phosphate (PMP). I found that mutation of Tyr265' to alanyl residue abolished the transaminase activity with L-alanine but retained that with D-alanine. The results suggest that Tyr265' serves as a catalytic base abstracting  $\alpha$ -hydrogen specifically from L-alanine.

In Chapter 4, I describe the results of x-ray crystallography of alanine racemase bound with *N*-(5'-phosphopyridoxyl)-D- or L-alanine, which are synthetic analogues of external Schiff base, one of the intermediates of the racemase reaction. The obtained results have confirmed that Lys39 and Tyr265' serve as the catalytic base specific to D- and L-alanine, respectively.

In Chapter 5, I describe the roles of Lys39 and Tyr265' in  $\alpha,\beta$ -elimination of  $\beta$ -chloro-D- and L-alanine catalyzed by alanine racemase. I found that Tyr265' is essential for the alanine racemization but not for the  $\alpha,\beta$ -elimination of  $\beta$ -chloro-D- and L-alanine.



Scheme 1-1. **Proposed mechanism of the alanine racemase reaction.** A, an internal aldimine of PLP with a lysyl residue; B, an external aldimine of PLP with D-alanine; C, a quinonoid intermediate formed after removal of  $\alpha$ -hydrogen from alanyl external aldimines B or D; D, an external aldimine of PLP with L-alanine.



## Chapter 2

### Chemical Rescue Studies of Lys<sup>39</sup>→Ala Mutant

#### INTRODUCTION

Alanine racemase [EC 5.1.1.1] is a pyridoxal 5'-phosphate (PLP) enzyme that occurs widely in bacteria and plays a central role in the metabolism of D-alanine, an essential component of the peptidoglycans in bacterial cell walls. General enzymological properties of alanine racemase have been studied in detail with the enzyme from *Bacillus stearothermophilus* (9-11).

The generally accepted mechanism of alanine racemase reaction is shown in Scheme 1-1 in General Introduction. The lysyl residue forming a Schiff base with PLP is shown to act as the base to abstract  $\alpha$ -hydrogen from a substrate amino acid in many PLP enzymes. The lysyl residue binding PLP in alanine racemase is also believed to play this role (5-7). However, the PLP-binding lysyl residue has another important function as a catalyst for transaldimination with a substrate amino acid. Therefore, one may expect complicated results by site-directed mutagenesis of the lysyl residue. However, Toney and Kirsch (12) have developed an elegant method named chemical rescue by which the function of a lysine residue lost by mutagenesis can be

rationally compensated by means of various kinds of amines. I have studied the role of lysine39 (Lys39), the PLP-binding lysyl residue of alanine racemase from *Bacillus stearothermophilus* (BSAR) (9-11), by chemical rescue studies of its Lys39→Ala (K39A) mutant alanine racemase with exogenous amines. I here show that Lys39 of BSAR probably serves as the base abstracting  $\alpha$ -hydrogen specifically from the D-enantiomer of alanine.

#### EXPERIMENTAL PROCEDURES

##### Materials

The K39A mutant alanine racemase was constructed (13) and purified to homogeneity as described below. D-Amino acid aminotransferase (14) were purified as described in literature. Alanine dehydrogenase was a gift from Dr. H. Kondo of Unitika Ltd., Osaka, Japan; L-lactate dehydrogenase was from Boeringer Mannheim.

##### Purification of Alanine Racemase

Purification steps of wild-type and mutant alanine racemases were described below. *Escherichia coli* JM109 cells were transformed with each prepared plasmid. Transformant cells were

cultivated in 5 l of Luria-Beritani's medium containing 50 µg/ml ampicillin at 37 °C for 10 h. The mutant enzyme was produced by induction by addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside 2 h after inoculation. Cells were harvested by centrifugation at 6,000 rpm for 10 min at 4 °C, and washed with 0.85% NaCl twice. Cells (about 10 g, wet weight) were suspended in 50 ml of 100 mM potassium phosphate buffer (pH 7.2) containing 20 µM PLP, 0.01% 2-mercaptoethanol, 0.1 mM phenylmethyl-sulfonyl fluoride, and 0.1 mM *p*-toluensulfonyl-L-phenylalanine chloromethyl ketone. The purification procedures described below were carried out at 4 °C unless otherwise specified. After the cell suspension was sonicated for 20 min, the lysate was centrifuged at 8,000 rpm for 20 min. The precipitate was resuspended in the same buffer, sonicated and centrifuged again. The supernatant solution was combined and incubated at 70 °C for wild-type enzyme (at 60 °C for mutant enzyme) for 60 min, cooled on ice, and then centrifuged at 8,000 rpm for 20 min. The supernatant solution was applied to a DEAE-TOYOPEARL 650M column (ϕ3.0 x 20 cm) equilibrated with buffer A which was 20 mM potassium phosphate buffer (pH 7.2) containing 0.01% 2-mercaptoethanol. The column was washed with 300 ml of buffer A, then the enzyme was eluted with a linear gradient from 0 to 200 mM KCl in buffer A. The active fractions were brought to 20% saturation of ammonium sulfate in buffer A and applied to a Butyl-

TOYOPEARL 650M column (ϕ3.0 x 20 cm) equilibrated with same buffer. The enzyme was eluted with a linear gradient from 20 to 0% saturation of ammonium sulfate in buffer A. The active fractions were dialysis against buffer A containing 20 µM PLP and then against buffer A without PLP. The dialysate was concentrated with a MILLIPORE Centriprep-10 concentrator. Purity of the enzyme was determined by SDS-PAGE.

### Preparation of [2-<sup>2</sup>H]-Alanine

D-[2-<sup>2</sup>H]-Alanine was prepared from a reaction mixture (1.0 ml) containing 0.1 M boric acid buffer (p<sup>2</sup>H 8.4), 89.1 mg of D-alanine, and 100 units of D-amino acid aminotransferase, whose solvent was replaced by <sup>2</sup>H<sub>2</sub>O by repeated concentrations and dilutions with 20 mM potassium phosphate buffer in <sup>2</sup>H<sub>2</sub>O (p<sup>2</sup>H 8.4) with Centricon 10 ultrafiltration unit. The reaction was performed at 37 °C for 16 h, and then stopped by heating at 100 °C for 10 min. After centrifugation, the supernatant solution was applied to a Dowex 50 (formate form) column (ϕ2.0 x 10 cm), and D-[2-<sup>2</sup>H]-alanine was eluted with 50 ml of 0.1 M HCl. The fractions containing D-alanine were pooled and evaporated to dryness.

L-[2-<sup>2</sup>H]-Alanine was prepared with L-methionine γ-lyase (15) in a mixture (p<sup>2</sup>H 7.5 in 1.0 ml <sup>2</sup>H<sub>2</sub>O) containing 89.1 mg of L-alanine and 23.7 mg of L-methionine γ-lyase. The reaction was carried out at

28 °C for 72 h, and then L-[2-<sup>2</sup>H]-alanine was isolated in the same manner as described above.

The deuterium contents of D-[2-<sup>2</sup>H]-alanine and L-[2-<sup>2</sup>H]-alanine were both higher than 97% when determined by <sup>1</sup>H-NMR.

### Protein Assays

Protein concentrations were determined by measurement of absorbance at 280 nm or by the method of Bradford (16) with bovine serum albumin as a standard. The absorption coefficients at 280 nm were estimated from the molecular weight and the amino acid composition of the enzymes.

### Enzyme Assays

Conversion of D-alanine to L-alanine catalyzed by BSAR was determined by following the formation of NADH in a coupled reaction with L-alanine dehydrogenase. The assay mixture contained 100 mM CAPS buffer whose pH was adjusted to pH 10.5 with tetramethylammonium hydroxide, 0.15 units of alanine dehydrogenase, 30 mM D-alanine, and 2.5 mM NAD<sup>+</sup> in a final volume of 1.0 ml. The reaction was started by addition of alanine racemase (about 0.01 µg) after pre-incubation of the mixture at 37 °C for 15 min. An increase in absorbance at 340 nm was followed. One unit of the enzyme was defined as the amount of enzyme that catalyzed

the racemization of 1 µmol of substrate per min.

D-Alanine formed from L-alanine was assayed with D-amino acid aminotransferase. The assay mixture contained 100 mM CAPS buffer (adjusted to pH 10.5 with tetramethylammonium hydroxide), 30 mM L-alanine, 5 mM α-ketoglutarate, 0.16 mM NADH, 12 units of D-amino acid aminotransferase, and 10 units of lactate dehydrogenase. The reaction was started by addition of alanine racemase (about 0.01 µg) after pre-incubation of the mixture at 37 °C for 10 min. A decrease in absorbance at 340 nm was monitored.

The amine-assisted reactions were conducted with about 52 µg of K39A mutant enzyme in the presence of various concentrations (0 - 200 mM) of amines, whose solutions were adjusted to pH 10.5 with tetramethylammonium hydroxide. The final ionic strength of the reaction mixture was adjusted to 0.5 by addition of tetramethylammonium chloride.

The apparent rate constant ( $k_{\text{obs}}$ ) of the reaction is expressed by the following equation (12):

$$k_{\text{obs}} = k_{\text{B}} [\text{amine}]_{\text{free}} + k_{\text{sol}} \quad (\text{i}),$$

where  $k_{\text{B}}$  is the rate constant of the reaction catalyzed by the deprotonated form of amine;  $k_{\text{sol}}$ , the rate constant of the reaction proceeding independently of amine; and  $[\text{amine}]_{\text{free}}$ , the concentration of deprotonated amine. Since  $[\text{amine}]_{\text{free}}$  depends on the ionization



constant ( $K_a$ ) of the amine and proton concentration  $[H^+]$  in the system, equation (i) is converted to equation (iv) as follows:

$$[\text{amine}]_{\text{protonated}} = [\text{amine}]_{\text{free}} \cdot [H^+] / K_a \quad (\text{ii})$$

$$\begin{aligned} [\text{amine}]_{\text{total}} &= [\text{amine}]_{\text{free}} + [\text{amine}]_{\text{protonated}} \\ &= [\text{amine}]_{\text{free}} (1 + [H^+] / K_a) \quad (\text{iii}) \end{aligned}$$

$$k_{\text{obs}} = k_B [\text{amine}]_{\text{total}} / (1 + [H^+] / K_a) + k_{\text{sol}} \quad (\text{iv}),$$

where  $[\text{amine}]_{\text{total}}$  is the concentration of added amine, and  $[\text{amine}]_{\text{protonated}}$  is the concentration of the protonated form of the amine.

The rate constant,  $k_B$ , was obtained from the plots of  $k_{\text{obs}}$  against  $[\text{amine}]_{\text{total}} / (1 + [H^+] / K_a)$ .

### Measurement of Isotope Effect

The substrate deuterium isotope effect was determined from the rates of racemization of D- or L-[2- $^2\text{H}$ ]-alanine in the presence of 100 mM methylamine. The conditions were the same as described above.

The solvent deuterium isotope effect was determined with the assay mixture (0.5 ml) that contained, in  $^2\text{H}_2\text{O}$ , various concentrations of D- or L-alanine, 100 mM CAPS buffer ( $p^2\text{H}$  10.9), and 100 mM methylamine. The reaction was started by addition of alanine racemase, which had been freed from  $\text{H}_2\text{O}$  by repeated lyophilization and dissolution in  $^2\text{H}_2\text{O}$ , and stopped by heating at 100 °C for 10 min. L-Alanine formed was determined as follows. A mixture (0.5 ml)

containing 100 mM CHES buffer (pH 9.0), 5 mM  $\text{NAD}^+$ , 0.15 units of alanine dehydrogenase, and an aliquot of the sample solution was incubated at 37 °C for 60 min, and then NADH formed was measured at 340 nm. D-Alanine produced was assayed in the same manner with a mixture containing 100 mM Tris-HCl buffer (pH 8.5), 0.2 mM NADH, 5 mM  $\alpha$ -ketoglutarate, 0.24 units of D-amino acid aminotransferase, 2.8 units of lactate dehydrogenase, and an aliquot of the sample solution. The incubation was done at 37 °C for 60 min.

## RESULTS

### Spectrophotometric Properties of K39A mutant enzyme

K39A mutant enzyme was identical with the wild-type BSAR in far-UV (180-200 nm) and near-UV (200-300 nm) CD spectra (data not shown). This suggests that K39A mutant enzyme has a secondary structure virtually identical to that of the wild-type enzyme. K39A mutant enzyme lacks Lys39 that inherently forms a Schiff base with PLP, but the enzyme showed an absorption maximum around 410 nm at pH 7.2, which maximum is slightly red-shifted in comparison with that of a free aldehyde form of PLP. This may have been due to the formation of a Schiff base between an unknown lysyl residue and PLP in K39A mutant enzyme, and I examined this possibility by incubating K39A mutant enzyme with 0.5 M  $\text{NaBH}_4$  at 25 °C for 8 h. The

absorption band at 410 nm disappeared and a new band appeared at around 330 nm, indicating that the bound PLP was reduced by the treatment. However, the PLP derivative was removed completely by dialysis against 6 M guanidine hydrochloride (data not shown). This indicates that the aldehyde group of PLP stays in a free form in K39A mutant enzyme. The mutant enzyme had no CD band due to the bound PLP, in clear contrast to the wild-type enzyme, which showed a negative CD band around 420 nm (Fig. 2-1).

#### **Effects of Primary Amines on K39A mutant enzyme**

K39A mutant enzyme was inactive, but its catalytic activities in both directions of racemization (from L- to D-alanine, and from D- to L-alanine) were restored by addition of 0.2 M methylamine. In contrast, the wild-type BSAR was inhibited by methylamine, though only slightly. A negative CD band similar to that of the wild-type BSAR appeared around 420 nm by addition of methylamine (Fig. 2-1). This suggests that methylamine forms a Schiff base with the C4' of PLP in the same manner as Lys39 of the wild-type BSAR. Shaw *et al.* (6) have reported that the position of PLP-binding lysine in BSAR is very similar to that in D-amino acid aminotransferase (17) upon superposition of their bound PLP. The two enzymes are similar in that their CD bands at around 420 nm due to bound PLP appear as

negative bands. X-ray crystallographic analysis has shown that the lysines in both enzymes approach the *re* face of bound PLP (6, 17). Therefore, methylamine forming a Schiff base with PLP in BSAR K39A presumably approaches the *re* face of PLP as well.



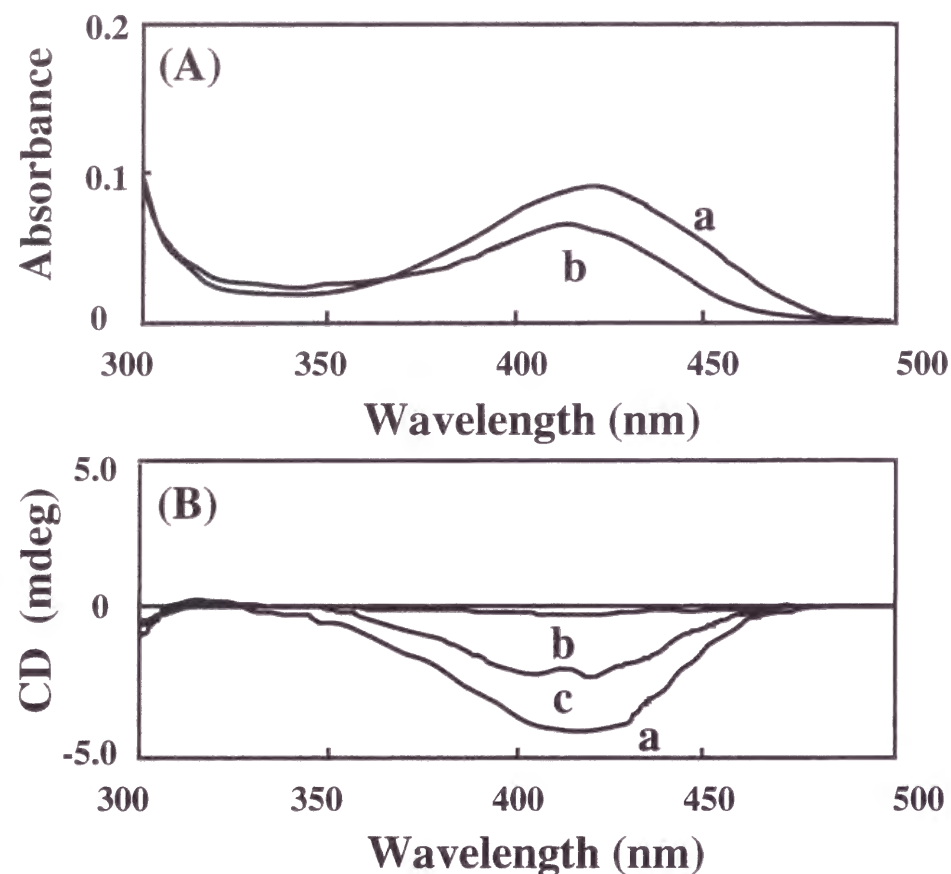


Fig. 2-1. **Absorption (A) and circular dichroism (B) spectra of the wild-type and the K39A mutant alanine racemases.** Absorption spectra of the wild-type (a) and K39A mutant (b) alanine racemases were taken in 20 mM potassium phosphate buffer (pH 7.2) containing 0.01% 2-mercaptoethanol at a protein concentration of 1.0 mg/ml. CD spectra of the wild-type enzyme (a), and the K39A mutant enzyme in the absence (b) or presence (c) of 500 mM methylamine were taken in 20 mM CAPS buffer (pH 10.5) containing 0.01% 2-mercaptoethanol at a protein concentration of 2.0 mg/ml with a JASCO J-600 recording spectropolarimeter at 25 °C with a 0.1 or 1.0-cm light path length cell under nitrogen atmosphere.

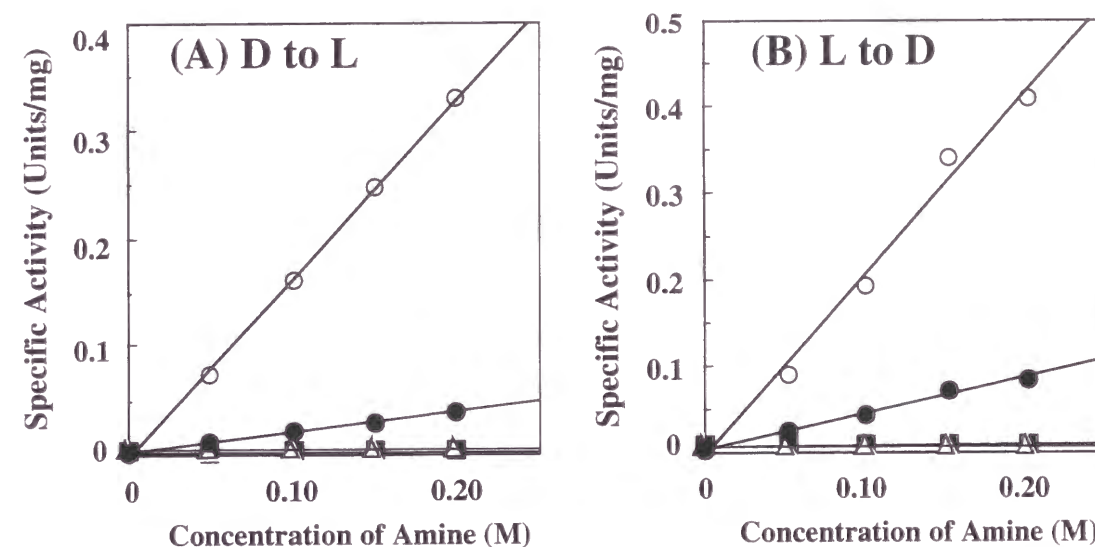


Fig. 2-2. **Amine-assisted racemization catalyzed by the K39A mutant enzyme.** The specific activities of the K39A mutant enzyme for the reactions from D- to L-alanine (A), and L- to D-alanine (B) were measured in the presence of various concentrations of methylamine at pH 9.0 (●) and 10.5 (○), dimethylamine at pH 10.5 (■), and diethylamine at pH 10.5 (Δ).

### Brønsted Analysis of the Amine-Assisted Racemization Catalyzed by BSAR K39A

The activities in both directions of alanine racemization catalyzed by K39A mutant enzyme increased proportionally with increases in the concentration of methylamine added (Fig. 2-2). I found that other alkylamines were also effective as catalysts (Figs. 2-3, 2-4), although the dialkylamines, dimethylamine and diethylamine were ineffective (Fig. 2-2). However, no evidence for saturation (at free base concentrations up to 200 mM) was observed, and there appeared to be no significant binding of alkylamines by K39A mutant enzyme under our experimental conditions. The rate of the methylamine-assisted reaction at pH 10.5 was much higher than that at pH 9.0 (Fig. 2-2). This indicates that only the free base of alkylamine participates in the catalysis, and I therefore calculated the rate constants of the reactions due to the free forms of amines ( $k_B$ ) on the basis of their dissociation constants ( $K_a$ ) and pH values of the reaction media, as described in the Experimental Procedures. Toney and Kirsch (12) have shown that a Brønsted analysis is applicable to the proton transfer catalyzed by K258A mutant aspartate aminotransferase assisted by exogenous amines, and found a multiple linear relationship between  $\log k_B$  and two independent factors, the  $pK_a$  and molecular volume of the amine, as follows:

$$\log k_B = \beta(pK_a) + V(\text{molecular volume}) + c,$$

where  $c$  is the constant term. I also found that a plot of  $\log k_B$  versus  $pK_a$  values of four kinds of amines with similar molecular volumes exhibited a linearity in both directions of alanine racemization (Fig. 2-3). The least squares fit of the data shown in Fig. 2-3 gave a Brønsted  $\beta$  value of 0.57 ( $R = 0.85$ ) for the  $D \rightarrow L$  reaction and of 0.62 ( $R = 0.92$ ) for the  $L \rightarrow D$  reaction. Moreover, I found that the  $\log k_B$  values were inversely proportional to the molecular volumes of a series of alkylamines with similar  $pK_a$  values (Fig. 2-4). The  $V$  values calculated from the data shown in Fig. 2-4 were  $-0.037 \text{ \AA}^{-3}$  ( $R=0.98$ ) for the  $D \rightarrow L$  reaction and  $-0.038 \text{ \AA}^{-3}$  ( $R= 0.99$ ) for the  $L \rightarrow D$  reaction.

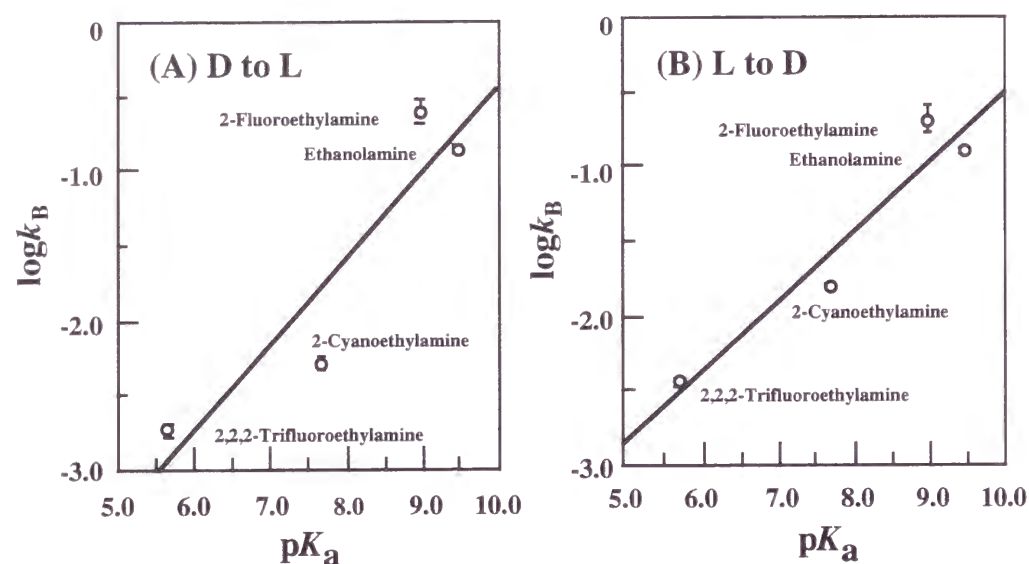


Fig. 2-3. Relationship between  $pK_a$  values of amines with similar molecular volumes and  $\log k_B$  of amine-assisted reactions from D- to L-alanine (A) and L- to D-alanine (B) catalyzed by K39A mutant enzyme. The  $pK_a$  values were taken from the previous report of Toney and Kirsch (12). The molecular volumes of amines reported (12) are as follows: ethanolamine,  $71.5 \text{ \AA}^3$ ; 2-fluoroethylamine,  $64.4 \text{ \AA}^3$ ; 2-cyanoethylamine,  $70.5 \text{ \AA}^3$ ; 2,2,2-trifluoroethylamine,  $71.6 \text{ \AA}^3$ .

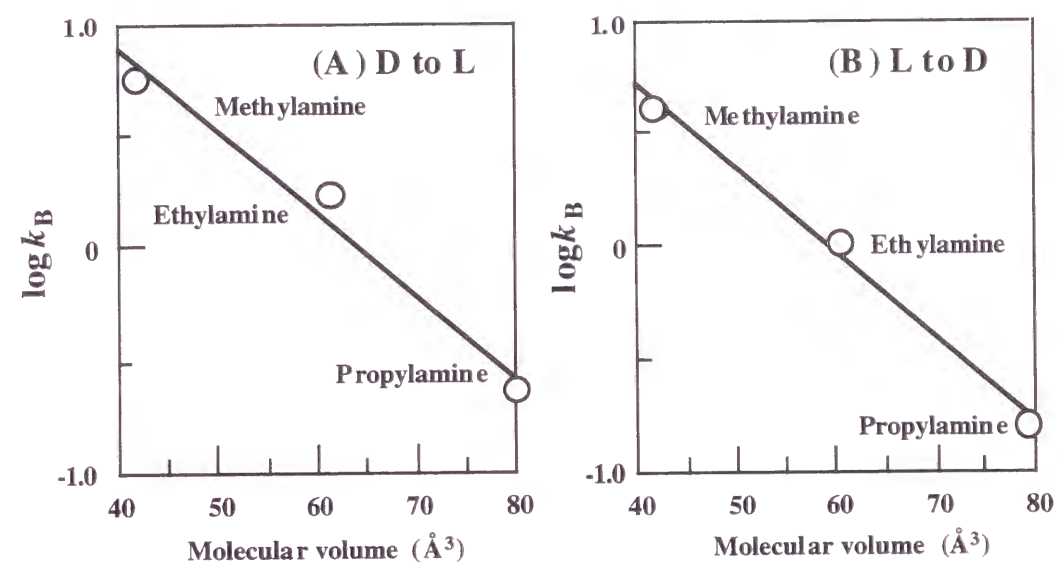


Fig. 2-4. Relationship between molecular volumes of amines with similar  $pK_a$  values and  $\log k_B$  of amine-assisted reactions from D- to L-alanine (A) and L- to D-alanine (B) catalyzed by K39A mutant enzyme. The molecular volumes were taken from the previous report of Toney and Kirsch (12). The  $pK_a$  values of amines reported (12) are as follows: methylamine, 10.6; ethanolamine, 10.6; propylamine, 10.5; butylamine, 10.6.



## Isotope Effect Studies

Faraci and Walsh (18) have demonstrated that the step of transaldimination is rate limiting in the reaction catalyzed by the wild-type BSAR. Therefore, the rescue effect by alkylamines observed here may be primarily due to their action as a base (or a nucleophile) in transaldimination. This is also supported by our findings of good agreement between the two Brønsted  $\beta$  values of the  $D \rightarrow L$  and the  $L \rightarrow D$  reactions. Faraci and Walsh (18) found that the isotope effect was virtually absent in the alanine racemization catalyzed by the wild-type BSAR. However, I examined the deuterium isotope effect in the methylamine-assisted racemization catalyzed by K39A mutant enzyme with D- and L-[2- $^2H$ ]-alanine. As shown in Fig. 2-5, I found a significant isotope effect for the reaction from D-alanine to L-alanine: a clear isotope effect was visualized on  $V$  rather than  $V/K$  (Fig. 2-5 and Table 2-1). However, no isotope effect was observed in the  $L \rightarrow D$  reaction. Therefore, the step of  $\alpha$ -deuteron abstraction from D-alanine was kinetically important and probably at least partially rate limiting throughout the whole process, while the removal of  $\alpha$ -deuteron from the antipode was kinetically insignificant. In marked contrast, however, when I examined the solvent isotope effect, a strong effect was observed only for the reaction from L-alanine to D-alanine (Table 2-1). Thus the step of  $\alpha$ -protonation (with deuterium)

to produce D-alanine is kinetically crucial, while its counterpart for the production of L-alanine from D-alanine is not. Presumably, the overall profile of the K39A mutant enzyme reaction assisted by an alkylamine is fairly asymmetric, as opposed to that of the wild-type BSAR reaction depicted by Faraci and Walsh (18).

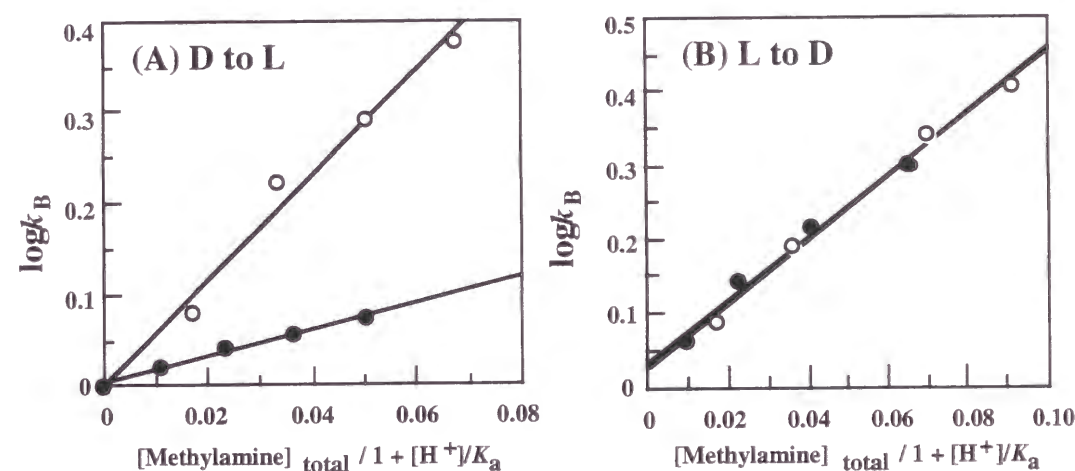


Fig. 2-5.  $\alpha$ -Deuterium substrate isotope effect on amine-assisted reactions catalyzed by K39A mutant enzyme. The apparent rate constants in the reactions from D to L-alanine (A) and L- to D-alanine (B) with non-labeled (○) or  $\alpha$ -deuterated D- or L-alanine (●) as substrates were plotted against the concentrations of deprotonated methylamine.

Table 2-1. Deuterium isotope effects of the methylamine-assisted racemization of D- and L-alanine catalyzed by the K39A mutant enzyme.

Reaction	Substrate isotope effect		Solvent isotope effect	
	$V_H/V^2H$	$(V/K)_H / (V/K)^2H$	$VH_2O/V^2H_2O$	$(V/K)_{H_2O} / (V/K)^2H_2O$
D- to L-alanine	5.4	2.6	1.3	1.0
L- to D-alanine	1.1	1.1	4.0	1.4

## DISCUSSION

Toney and Kirsch (12) developed a general approach in which the activity of a mutant enzyme lacking a catalytically important residue can be restored by the addition of small molecules functionally equivalent to the missing catalytic group. Since their report, this "chemical rescue" approach has been successfully applied to a number of enzymes to identify catalytic residues and to establish structure-activity relationships. The rescuer molecules used are amines for lysine-mutated enzymes (19-21), guanidines for arginine-mutated enzymes (22, 23), phenol for a tyrosine-mutated enzyme (24), azide

or formate for a glutamate-mutated enzyme (25), and imidazole for histidine-mutated enzymes (26, 27). In this study, I have demonstrated by combination of chemical rescue studies and isotope effect analysis of the amine-assisted reactions catalyzed by K39A mutant enzyme that Lys39 of the wild-type BSAR probably mediates not only transaldimination but also  $\alpha$ -hydrogen abstraction from the substrate. This is one of the rare examples of a chemical rescue study clarifying multiple functions of a catalytically important residue by combination with isotope effect analysis.

*DadB* and *alr* alanine racemases from *Salmonella typhimurium* are distinct from BSAR in that the former enzymes show substrate and solvent deuterium isotope effects (18). Abstraction of  $\alpha$ -deuteron from D-[2- $^2H$ ]-alanyl-PLP aldimine and protonation (with deuteron) of the carbanion intermediate (Scheme 1-1 in Chapter 1) to give the D-alanyl aldimine in  $^2H_2O$  are rate limiting (though maybe only partially) in the racemization reaction catalyzed by the *Salmonella* enzymes. Although the isotope-effect values vary widely among these enzymes, the amine-assisted reaction catalyzed by K39A mutant enzyme is identical to the reactions catalyzed by the *Salmonella* enzymes in this respect.

Sawada *et al.* (4) have proposed on the basis of kinetic evidence that the reaction catalyzed by BSAR proceeds through a two-base mechanism. Shaw *et al.* (6) have postulated on the basis of x-ray



crystallographic studies that Lys39 and Tyr265' of BSAR serve as the bases to abstract  $\alpha$ -hydrogen from the alanyl-PLP aldimine and to protonate the carbanion intermediate to give the alanyl-PLP aldimine. After  $\alpha$ -hydrogen is abstracted from the alanyl-PLP aldimine by one base, the other base, which lies on the opposite side of the plane of the alanyl-PLP carbanion intermediate, probably protonates the carbanion in order for racemization to occur. Tyr265' is the only possible residue whose side chain lies almost directly opposite the side chain of Lys39, and the hydroxyl group of Tyr265' is postulated to serve as the second base (6). The chemical rescue studies presented here strongly suggest that Lys39 serves as the base that abstracts  $\alpha$ -hydrogen from the D-alanyl-PLP aldimine and protonates the carbanion intermediate to produce the PLP-aldimine of the same enantiomer. Therefore, Tyr265' is assumed to be the second base specifically acting on the L-alanyl-PLP aldimine. The crystal structure of BSAR complex with (*R*)-1-aminoethylphosphonic acid (L-Ala-P) (7), a tight-bind inhibitor of BSAR (28), has demonstrated that the phenolic oxygen of Tyr265' is located at a position much closer than the NZ of Lys39 to the  $\alpha$ -carbon of the L-Ala-P-PLP aldimine: the phenolic oxygen of Tyr265' is appropriately aligned for proton abstraction from an L-isomer in the active-site of the clarified structure.

Both Lys39 and Tyr265' are conserved in both *dad B* and *alr* alanine racemases of *S. typhimurium* (29, 30), and these residues probably play the same roles, respectively, as those in BSAR. According to the kinetic isotope effect studies of Faraci and Walsh (18), I can assume that Lys39 is less effective than Tyr265' as the base in the *S. typhimurium* enzymes. The only marked difference between BSAR and the *S. typhimurium* enzymes is that Gln314' of BSAR is replaced by a methionine in both *S. typhimurium* enzymes. Crystallographic studies (6) have suggested that Gln314' of BSAR plays an important role at the active-site. Gln314' of BSAR is hydrogen bonded with a water molecule, which is hydrogen-bonded to the O3' of the PLP ring. Moreover, the side-chain OE1 of Gln314' is also hydrogen-bonded with NH1 of Arg136, which is believed to be the binding site for the carboxyl group of the substrate alanine. The lack of a glutamine at this position in the *S. typhimurium* enzymes may be responsible for the apparently higher efficiency of Lys39 than Tyr265' as a base to abstract  $\alpha$ -hydrogen from the substrate alanine. Replacement of Lys39 with alanine probably perturbed the active-site structure of K39A mutant enzyme even in the presence of the rescuer alkylamines, and this may be the reason that the  $\alpha$ -hydrogen-abstraction step was rate limiting (though maybe only partially) in the amine-assisted reaction.

According to the general mechanism of transaldimination, the

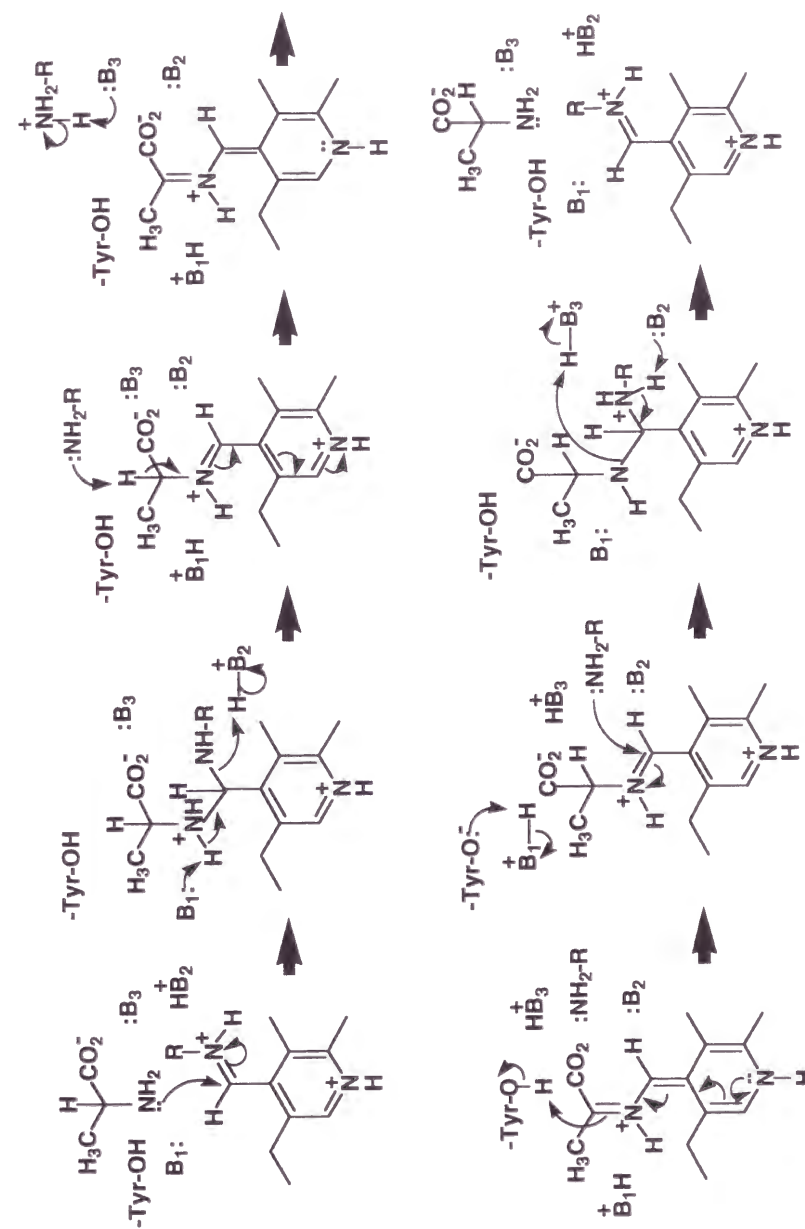
imino nitrogen of an internal Schiff base is considered to be non-protonated and to remove a proton from the protonated form of the amino group of a substrate amino acid. Thus, the deprotonated amino group of the amino acid is allowed to attack the 4'-carbon of the internal Schiff base. The PLP-binding lysyl residue is released from the geminal diamine, and the first transaldimination is accomplished. The second transaldimination proceeds through a nucleophilic attack on the 4'-carbon of the external Schiff base by the free amino group of the lysyl residue to release a product. The PLP-binding lysyl residue is known to be crucial as the catalyst in product release, as shown by site-directed mutagenesis studies on aspartate aminotransferase (12), tryptophan synthase (31), D-amino acid aminotransferase (32, 33), and aromatic L-amino acid decarboxylase (34); the product (or substrate) forms a stable Schiff base with PLP and is not released readily from the enzyme unless the PLP-binding lysine is present. On the basis of the mechanism described above, I propose a mechanism of the amine-assisted reaction catalyzed by K39A mutant enzyme in which three bases mediate the proton transfer by cooperation with Lys39 and Tyr265' (Scheme 2-1). B<sub>1</sub> transfers a proton from the amino nitrogen of the geminal diamine intermediate to the phenolate of Tyr265'; B<sub>2</sub> protonates and deprotonates the alkylamine moiety of the geminal diamine intermediate; B<sub>3</sub> transfers a proton from an alkylamine to the amino acid moiety released from the

geminal diamine intermediate. B<sub>1</sub>, B<sub>2</sub> and B<sub>3</sub> are possibly His166, Arg136 and Asp313', respectively, according to the x-ray structures of BSAR (6, 7), although this hypothesis can not be proven definitively until the x-ray structure of alanine-bound BSAR is determined.

All monoalkylamines (and their derivatives) examined here showed significant rescue effects to enhance the activity of K39A mutant enzyme, and their efficiencies depended on both molecular volume and  $pK_a$ . However, dialkylamines were ineffective, and in this respect our results differ markedly from those of Kirsch and Toney (35); that is, I found that dimethylamine was also an effective rescuer for the K258A mutant of aspartate aminotransferase. This difference was probably due to a difference in the catalytic role of rescuer amines in the enzymes examined. Amines primarily serve as the base that abstracts  $\alpha$ -hydrogen from substrate amino acid in aspartate aminotransferase. In K39A mutant enzyme, however, transaldimination is kinetically more important than  $\alpha$ -hydrogen abstraction as a role of amines: transaldimination is believed to be rate determining in the wild-type BSAR reaction (18). Dialkylamines are probably less efficient than monoalkylamines as a catalyst of transaldimination for reason of steric hindrance, since amines need to bind covalently to C4' of PLP in transaldimination. However, no covalent bond formation is required in order for amines to abstract



$\alpha$ -hydrogen from substrate. The Brønsted  $\beta$  values observed for both the  $D \rightarrow L$  and  $L \rightarrow D$  reactions probably reflect the transition state of either of the two steps in transaldimination: either deprotonation of the protonated form of amino acid substrate by alkylamine to form PLP-aldimine or nucleophilic addition of alkylamine at C4' of the substrate-PLP aldimine. Both of these steps depend on the  $pK_a$  values of the amines used (36), and I currently have no explanation for the Brønsted  $\beta$  values I obtained. Nonetheless, this constitutes the first report of a chemical rescue for a lysyl residue responsible for transaldimination.



Scheme 2-1. **Proposed mechanism of amine-assisted alanine racemization reactions catalyzed by K39A mutant enzyme.** B<sub>1</sub>, B<sub>2</sub> and B<sub>3</sub> are the side chains of amino acid residues presumably participating in proton donation and abstraction. The phenolic hydroxyl group of Tyr265' is assumed to be the second base abstracting  $\alpha$ -hydrogen from alanyl-PLP aldimine and donating a proton to the  $\alpha$ -position of the carbanion intermediate.

## SUMMARY

The lysine residue binding with the cofactor pyridoxal 5'-phosphate (PLP) plays an important role in catalysis, such as in the transaldimination and abstraction of  $\alpha$ -hydrogen from a substrate amino acid in PLP-dependent enzymes. I have studied the role of Lys39 of alanine racemase [EC 5.1.1.1] from *Bacillus stearothermophilus*, the PLP-binding residue of the enzyme, by replacing it site-specifically with alanine and characterizing the resultant K39A mutant enzyme. The mutant enzyme turned out to be inherently inactive, but gained an activity as high as about 0.1% of that of the wild-type enzyme upon addition of 0.2 M methylamine. The amine-assisted activity of the mutant enzyme depended on the  $pK_a$  values and molecular volumes of the alkylamines used. A strong kinetic isotope effect was observed when  $\alpha$ -deuterated D-alanine was used as a substrate in the methylamine-assisted reaction, but little effect was observed using its antipode. In marked contrast, only L-enantiomer of alanine showed a solvent isotope effect in deuterium oxide in the methylamine-assisted reaction. These results suggest that methylamine serves as a base not only to abstract the  $\alpha$ -hydrogen from D-alanine but also to transfer a proton from water to the  $\alpha$ -position of the deprotonated (achiral) intermediate to form D-alanine.

Therefore, the exogenous amine can be regarded as a functional group fully representing Lys39 of the wild-type enzyme. Lys39 of the wild-type enzyme probably acts as the base catalyst specific to the D-enantiomer of alanine. Another residue specific to the L-enantiomer in the wild-type enzyme is kept intact in the K39A mutant enzyme.

## Chapter 3

### Tyrosine265' Serves as a Base Abstracting $\alpha$ -Hydrogen from L-Alanine

#### INTRODUCTION

Alanine racemase reaction probably proceeds through the following steps (18). PLP bound with the active-site lysyl residue through an internal Schiff base (Fig. 3-1, A) reacts with a substrate to form an external Schiff base (B). The subsequent  $\alpha$ -hydrogen abstraction results in the formation of a resonance-stabilized deprotonated intermediate (C). If reprotonation occurs at the C2 of the substrate moiety on the opposite face of the planar intermediate (C), then an antipodal aldimine (D) is formed. An isomerized amino acid and a PLP form of the enzyme (E) are generated by the subsequent transaldimination between PLP-binding lysyl residue and intermediate (D). The kinetic analyses (5, 37) and the x-ray crystallographic studies (6-8) of the alanine racemase from *Bacillus stearothermophilus* have suggested that the reaction proceeds through a two-base mechanism with lysine39 (Lys39) and tyrosine265' (Tyr265') from other subunit as catalytic residues. These two residues are situated on each side of the pyridine ring of PLP and

seem to serve as proton donors and proton acceptors in order to accomplish removal and return of the  $\alpha$ -hydrogen of the substrate amino acids. Lys39 bears an important role: it mediates transaldimination to facilitate the formation of PLP-aldimine of a substrate amino acid as well as an internal Schiff base (Fig. 3-1, A  $\rightleftharpoons$  B; D  $\rightleftharpoons$  E). Through chemical rescue studies with Lys39 $\rightarrow$ Ala (K39A) mutant enzyme, I showed that Lys39 also serves as a catalytic base specifically directing to D-enantiomer of substrate amino acid: it abstracts  $\alpha$ -hydrogen from D-alanine and transfers a proton from water to the C-2 of the deprotonated (achiral) intermediate to form D-alanine (37). Sun and Toney sustained the speculation that Tyr265' serves as a catalytic base through the study of the site-directed mutagenesis of arginine219 (Arg219) interacting with pyridine nitrogen of PLP (5). Mutation of Arg219 interacting with Tyr265' via hydrogen-bonding network affects the kinetic isotope effect in the  $\alpha$ -hydrogen transfer from (or to) L-alanine specifically.

In this chapter, I describe the reaction catalyzed by the Tyr265' $\rightarrow$ Ala (Y265A) mutant enzyme, and obtained a direct evidence that Tyr265' abstracts  $\alpha$ -hydrogen from L-alanine and transfers a proton to the C-2 of the deprotonated intermediate to form D-alanine. I previously demonstrated that alanine racemase catalyzes the transamination as a side reaction (13). Prolonged incubation of the enzyme with D- or L-alanine resulted in the spectral change of the



enzyme suggesting the formation of the pyridoxamine (PMP) form of enzyme with a concomitant inactivation of the enzyme. The activity was recovered by the incubation with  $\alpha$ -keto acids such as pyruvate and  $\alpha$ -ketobutyrate. Transamination catalyzed by alanine racemases is probably attained through a sequence  $A \rightarrow B \rightarrow C \rightarrow F$  (or  $G$ )  $\rightarrow H$  (Fig. 3-1). An equivalent route can be delineated for the antipode:  $E \rightarrow D \rightarrow C \rightarrow G$  (or  $F$ )  $\rightarrow H$ . If Tyr265' is a catalytic residue mediating  $\alpha$ -hydrogen transfer from (or to) L-alanine, mutation of the residue should abolish the both direction of racemization ( $A \rightarrow B \rightarrow C \rightarrow D \rightarrow E$  and  $E \rightarrow D \rightarrow C \rightarrow B \rightarrow A$ ) and transamination with L-alanine ( $E \rightarrow D \rightarrow C \rightarrow G$  (or  $F$ )  $\rightarrow H$ ), but retain the transamination activity with D-alanine ( $A \rightarrow B \rightarrow C \rightarrow F$  (or  $G$ )  $\rightarrow H$ ). I have studied the first half transamination, conversion of PLP to PMP, catalyzed by the wild-type and mutant alanine racemases through the spectral change of the enzyme. I have also studied the effect of mutation of Tyr265' on the latter half transamination, from PMP and pyruvate, by analyzing the  $^3\text{H}$  release with stereospecifically labeled  $[4'-^3\text{H}]\text{PMP}$ .

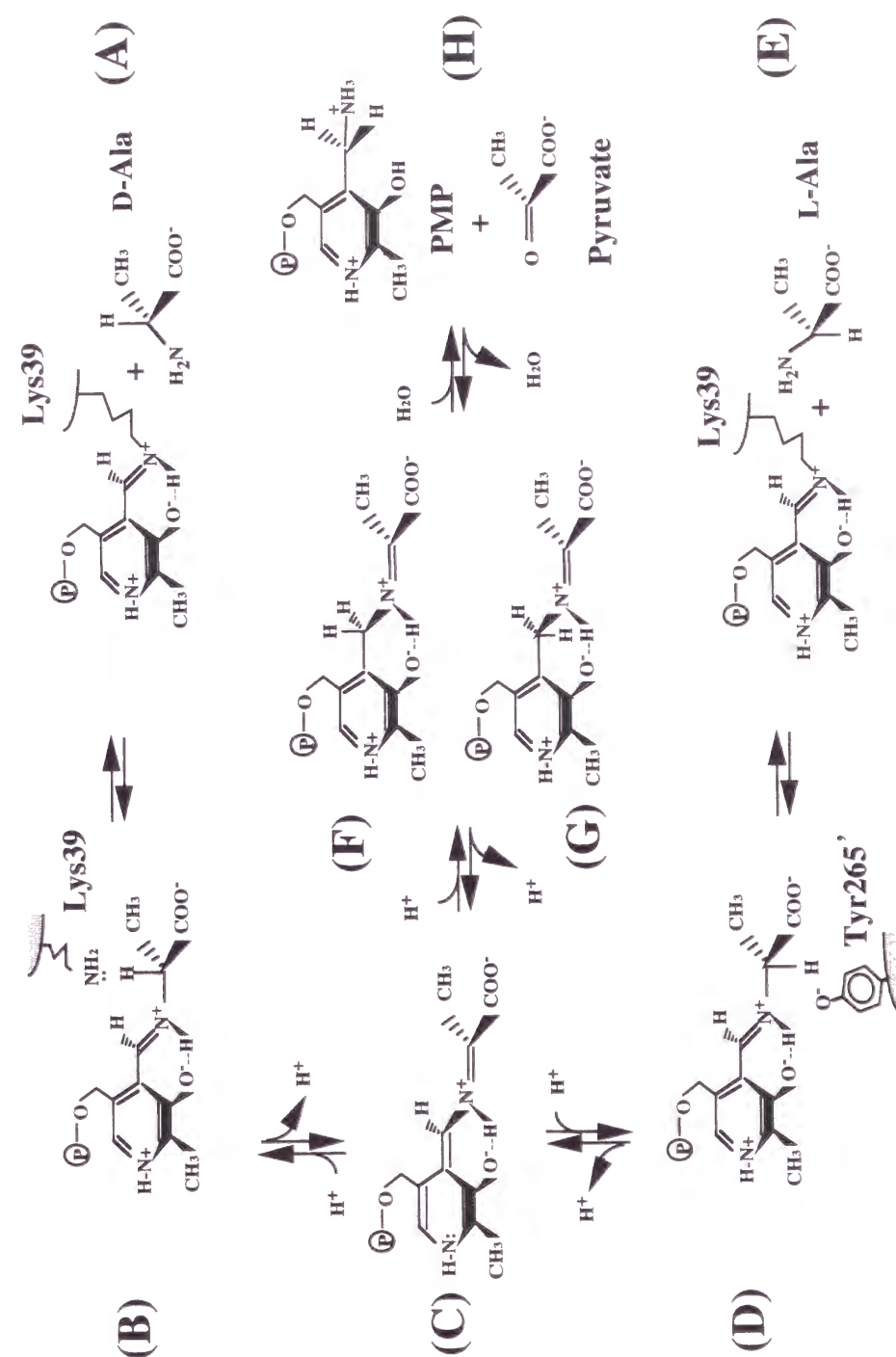


Fig. 3-1. Reaction mechanism of alanine racemase.

## EXPERIMENTAL PROCEDURES

### Materials

The plasmid pAR310 carrying the alanine racemase gene from *B. stearothermophilus* was prepared as described previously (13). The oligonucleotides used for mutagenesis were synthesized by means of phosphoamidite chemistry. The enzymes for DNA manipulation were purchased from Takara Shuzo, Kyoto, Japan. L-Alanine dehydrogenase was a gift from Dr. H. Kondo of Unitika (Osaka, Japan). D-Amino acid aminotransferase was prepared as described previously (14). Branched chain L-amino acid aminotransferase (BCAT) of *Escherichia coli* K-12 was kindly supplied by Professor H. Kagamiyama and Dr. K. Inoue of Osaka Medical College (38). Aspartate aminotransferase (AspAT) from porcine heart was obtained from Sigma (U.S.A.). L-Lactate dehydrogenase was purchased from Boeringer Mannheim (Germany). All other reagents and chemicals were of analytical grade.

### Site-directed mutagenesis

Site-directed mutagenesis was carried out by Kunkel's method (39). The four mutagenic primers used were 5'-CGTCGCACCA GCGCTCACCTTTTCGCC-3' for preparation of Y265A mutant

enzyme, 5'-CGTCGCACCAAAAGCTCACCTTTTCGCC-3' for Tyr265'→Phe (Y265F) mutant enzyme, 5'-CGTCGCACCACTGCTCACCTTTTCGCC-3' for the Tyr265'→Ser (Y265S) mutant enzyme, and 5'-CGCAGTCGCAAAAGCCGTGTACAACCCTTC-3' for His166→Ala (H166A) mutant enzyme. Mutation was confirmed by DNA sequencing by the dye deoxy terminator method with an Applied Biosystems Model 373A automated DNA sequencer.

### Purification of the Wild-type and Mutant Enzymes

The wild-type and mutant enzymes of BSAR were overproduced in recombinant *E. coli* JM109 cells and purified by heat-treatment, DEAE-TOYOPEARL 650M, and Butyl-TOYOPEARL 650M column chromatography as described in Chapter 2. Purity of the enzymes were determined by SDS-PAGE.

### Spectrophotometric Measurements

Absorption spectra were taken with a Shimadzu MPS-2000 or a Beckman DU-640 spectrophotometers.

### Assays

Protein concentrations were determined as described in Chapter 2. The enzymatic activity of racemization was determined as described in Chapter 2. Conversion of PLP form of enzyme to PMP

form was assayed by following the absorption spectral change of the enzyme in the reaction mixture containing 0.1 M potassium phosphate buffer (pH 7.2), 0.1 M D- or L-alanine and each 1 mg of the wild-type, Y265A or K39A mutant alanine racemases in a final volume of 1.0 ml. The reaction was started by addition of D- or L-alanine and carried out at 25 °C.

### Preparation of apoenzymes

The wild-type and mutant enzymes were dialyzed against 10 mM potassium phosphate buffer (pH 7.2) containing 50 mM hydroxylamine and 0.01% 2-mercaptoethanol for 24 h at 4 °C, followed by dialysis against the same buffer without hydroxylamine overnight. Ratio of the formation of each apoenzyme was determined by measurement of the activity in the presence or absence of 20 µM PLP. Activity of the K39A mutant enzyme was assayed in the presence of 0.2 M methylamine. Apo-AspAT and apo-BCAT were prepared as described previously (40).

### Preparation of (4'S)- and (4'R)-[4'-<sup>3</sup>H]PMP

(4'S)- and (4'R)-[4'-<sup>3</sup>H]PMP were prepared by incubation of randomly labeled [4'-<sup>3</sup>H]PMP with apo-BCAT and apo-AspAT, respectively (40). The reaction mixture (550 µl) for the preparation of (4'R)-[4'-<sup>3</sup>H]PMP contained 0.1 M Tris-HCl buffer (pH 8.0),

0.05% sodium azide, 19 nmol of [4'-<sup>3</sup>H]PMP, and 40 nmol of apo-AspAT. That for (4'S)-[4'-<sup>3</sup>H]PMP contained 45 nmol of [4'-<sup>3</sup>H]PMP, and 60 nmol of apo-BCAT. The reaction was carried out at 32 °C for 18 h, and stopped by addition of HCl to the final concentration of 0.2 M. The reaction mixtures for the preparation of S- and R-isomers were mixed with 1.9 and 4.5 nmols of unlabeled PMP, respectively, and further incubated at 32 °C for 2 h. After centrifugation, each supernatant was dried with a Speed Vac Concentrator (Savant, U.S.A.), and the residue was dissolved in 0.2 ml of H<sub>2</sub>O. PMPs were purified by a reverse phase column chromatography (Waters Ultron S-C<sub>18</sub> column) with Shimadzu LC-6A HPLC system as described previously (40). Radioactivity was determined with a Beckman LS-6500 scintillation counter with using 10 ml of Clear-sol II (Nacalai Tesque, Japan) as a scintillator. The specific radioactivities of prepared (4'S)- and (4'R)-[4'-<sup>3</sup>H]PMP were 0.84 × 10<sup>6</sup> and 1.32 × 10<sup>6</sup> dpm / µmol, respectively.

### Stereospecificity for the hydrogen abstraction from PMP during the transamination between PMP and pyruvate

The reaction mixture (0.1 ml) contained 100 mM CAPS buffer (pH 10.0), 0.5 nmol of (4'S)- or (4'R)-[4'-<sup>3</sup>H]PMP, 10 nmol of sodium pyruvate, 0.05% sodium azide, and each 2.0 nmol of apo-



wild-type, apo-K39A, or apo-Y265A mutant enzyme. The reaction was started by addition of pyruvate after pre-incubation of the mixture at 32 °C for 30 min. The reaction was carried out at 32 °C for 18 h, stopped by addition of HCl to a final concentration of 0.2 M, and dried with a Speed Vac Concentrator. The residue was dissolved in 0.2 ml of H<sub>2</sub>O, and the radioactivity was determined as described above.

## RESULTS

### Mutation of Tyr265'

I previously showed that the rates of D → L and L → D reactions catalyzed by Y265A mutant enzyme were 0.06 and 0.03% of those of the wild-type enzyme, respectively (41). Because Y265A mutant enzyme still catalyzes the α,β-elimination of β-chloro-D- and L-alanine with the similar kinetic parameters to those of the wild-type enzyme, loss of the racemase activity was not derived from the distortion of the gross conformation of the enzyme (41). To confirm the role of Tyr265', I converted the residue to phenylalanyl and seryl residues in addition to alanyl residue. As shown in Table 3-1, specific activities of D → L and L → D reactions catalyzed by Tyr265' mutant enzymes were about 3 - 4 orders of magnitude less than those catalyzed by the wild-type enzyme, suggesting that Tyr265' plays an

important role in alanine racemization.

Table 3-1. Activities of the wild-type, Y265A, Y265F, Y265S, and H166A mutant alanine racemases.

	D- to L-Ala		L- to D-Ala	
	specific activity (units/mg)	ratio (%)	specific activity (units/mg)	ratio (%)
wild-type	628	100	942	100
Y265A	0.12	0.02	0.24	0.03
Y265F	0.01	0.00	0.01	0.00
Y265S	0.28	0.05	0.36	0.04
H166A	95.2	15	151.2	16

### Transamination between PLP and both enantiomers of alanine catalyzed by the wild-type and mutant alanine racemases

If Tyr265' is the catalytic residue abstracting 2-hydrogen from L-alanine and returning proton to C-2 of the deprotonated intermediate to form L-alanine (reaction between (C) and (D) in Fig. 3-1), mutation of Tyr265' abolishes both directions of racemization.

The mutation should also abolish the transamination activity with L-alanine, but retain that with D-alanine. Because, transamination with D-alanine does not proceed the pathway between (C) and (D) (Fig. 3-1). When the wild-type enzyme was incubated with D- or L-alanine, absorption maximum at 420 nm gradually decreased with increase in that at 330 nm (Fig. 3-2). Kinetics of the decrease in absorbance at 420 nm was biphasic. The first phase followed the pseudo-first order kinetics, and the second phase was too slow to be analyzed kinetically (data not shown). The apparent rate constants of the decrease in absorbance at 420 nm of the wild-type enzyme with L- and D-alanine were nearly the same, -0.0059 and -0.0063 ( $\text{min}^{-1}$ ), respectively. When Y265A mutant enzyme reacted with D-alanine, decrease in absorption maximum at 420 nm and increase in that at 330 nm proceeded more rapidly. Apparent rate constant of the decrease in absorbance at 420 nm was -0.036, which was 5.7 times higher than that obtained in the reaction of the wild-type enzyme and D-alanine. In contrast, rate of the absorption spectral change of Y265A mutant enzyme caused by the addition of L-alanine was extremely slow: about 90% of the initial peak height at 420 nm remained after 12-h incubation (Fig. 3-2). These results demonstrate that mutation of Tyr265' abolishes the transamination activity with L-alanine but retains that with D-alanine. This is compatible with the hypothesis that Tyr265' is the base catalyzing the abstraction and addition of 2-

hydrogen of L-alanine. The K39A mutant enzyme catalyzed no transamination with either D- or L-alanine as a substrate (data not shown). This is probably because the mutation disabled the transaldimination (reactions between (A)  $\rightarrow$  (B), and (E)  $\rightarrow$  (D) in Fig. 3-1).



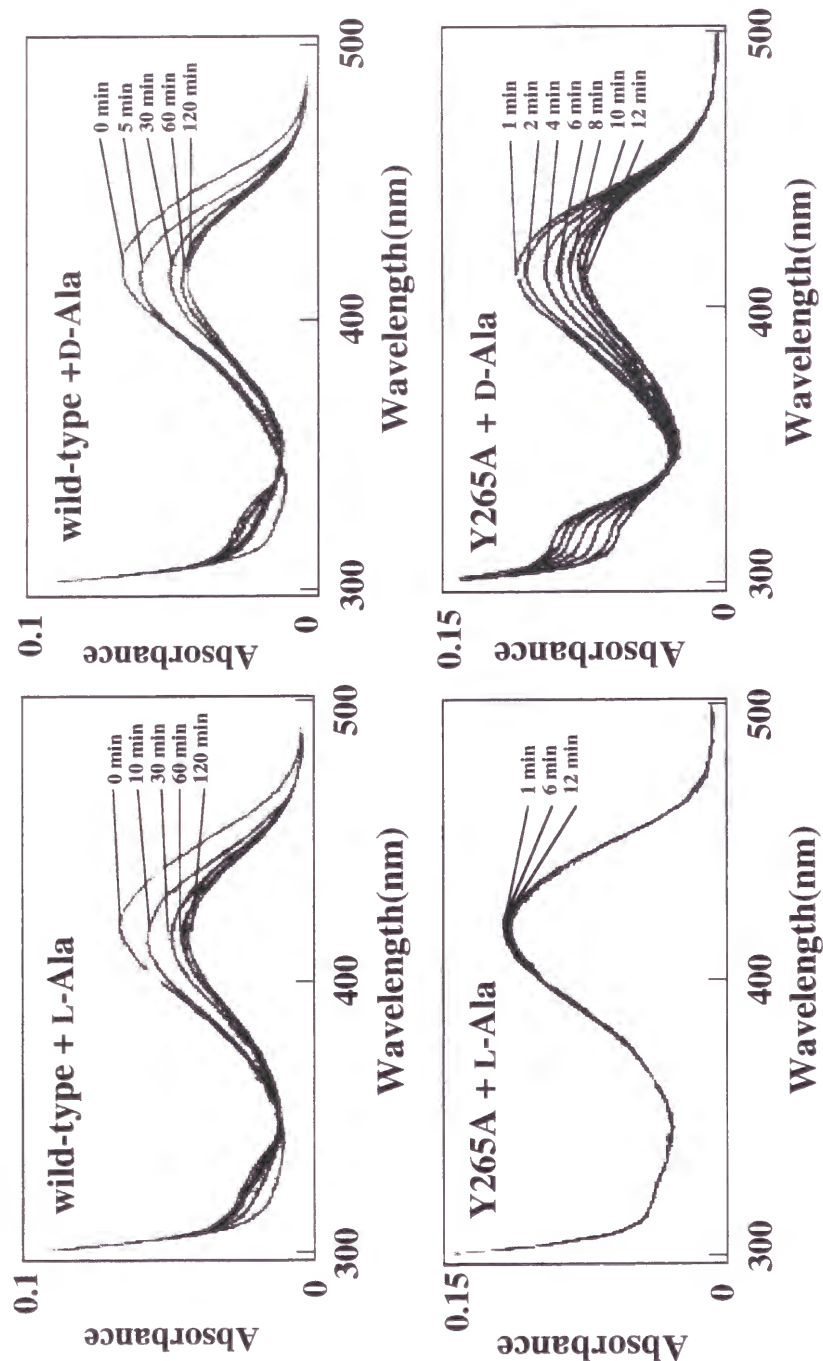


Fig. 3-2. Spectral change of the wild-type and Y265A mutant alanine racemases during incubation with L- and D-alanine. The UV-visible spectra (500 - 300 nm) were taken at the indicated times.

### Stereospecificity for the hydrogen abstraction from C-4' carbon of PMP catalyzed by the wild-type and mutant alanine racemase

During the latter half reaction of transamination, transamination from PMP to keto acid, one of the two hydrogens at C-4' of PMP is abstracted and transferred to the C-2 of the keto acid. We have been studying the stereospecificity for the hydrogen abstraction from C-4' with stereospecifically labeled [4'-<sup>3</sup>H]PMP. The hydrogen abstraction catalyzed by aminotransferases so far studied is stereospecific: D-amino acid aminotransferase and BCAT catalyze the abstraction from the 4'*R* isomer of [4'-<sup>3</sup>H]PMP; the other aminotransferases, from 4'*S* isomer (40, 42). The lysyl residue bound to PLP mediates the abstraction and transfer of the C-4' hydrogen in the aspartate aminotransferase (43, 44) and D-amino acid aminotransferase reactions (32, 45). However, only a small portion of the abstracted tritium is transferred to the amino acceptor, and the rest is released into solvent due to an exchange with solvent hydrogen (40). Therefore, I can readily determine the stereospecificity by measuring the radioactivity released into solvent. In contrast to amino transferases, amino acid racemase with low substrate specificity and alanine racemase catalyze the non-stereospecific hydrogen abstraction (46, 47). These enzymes catalyzed the release of tritium from both

4'S and 4'R enantiomers of [4'-<sup>3</sup>H]PMP into solvent in the presence of pyruvate. Non-stereospecific hydrogen abstraction probably reflects that the racemase reaction proceeds through two-base mechanism in contrast to the aminotransferase reaction proceeding through one-base mechanism. If this is the case, mutation of one of the catalytic residues should make the tritium abstraction catalyzed by racemase stereospecific.

In this experiment, I prepared (4'R)-[4'-<sup>3</sup>H]PMP and (4'S)-[4'-<sup>3</sup>H]PMP by making use of the stereospecific exchange of (4'S)- and (4'R)-<sup>3</sup>H of the randomly labeled [4'-<sup>3</sup>H]PMP with the solvent hydrogen catalyzed by apo-AspAT and apo-BCAT, respectively (40). Stereospecificity of the label of the prepared PMPs were confirmed by the reaction with apo-AspAT and apo-BCAT (Table 3-2). When apo form of the wild-type alanine racemase reacted with each (4'R)-[4'-<sup>3</sup>H]PMP and (4'S)-[4'-<sup>3</sup>H]PMP, tritium was released from both enantiomers (Table 3-2). Amount of the tritium released from (4'R)-[4'-<sup>3</sup>H]PMP was higher than that from the S-isomer. I obtained the similar results in our previous experiments (47). The phenomenon is probably derived from the difference between the rate of abstraction of C-4' pro-R hydrogen and that of pro-S hydrogen at C-4' of PMP catalyzed by alanine racemase. Apo-Y265A mutant enzyme abstracted the comparable amount of tritium from (4'R)-[4'-<sup>3</sup>H]PMP to that released in the reaction with the wild-type enzyme. In

contrast, amount of the tritium released from (4'S)-[4'-<sup>3</sup>H]PMP by apo-Y265A mutant enzyme was only one sixth of that by the wild-type enzyme (Table 3-2). Abstraction of tritium from [4'-<sup>3</sup>H]PMP catalyzed by the alanine racemase became R-specific by the conversion of Tyr265' to alanyl residue. These results indicate that Tyr265' serves as a catalytic residue abstracting the pro-S hydrogen at C-4' of PMP ( (G) → (C) in Fig. 3-1). Little or no tritium was released from either enantiomer of PMP by incubation with K39A mutant enzyme and pyruvate (Table 3-2). This can be explained by assuming that Lys39 assists the ketimine formation from PMP and pyruvate ( (H) → (G) or (F) in Fig. 3-1) and (or) tritium abstraction from (4'S)-[4'-<sup>3</sup>H]PMP catalyzed by Tyr265' ((G) → (C) in Fig. 3-1).

Table 3-2. Release of tritium from stereospecifically labeled [4-<sup>3</sup>H]PMPs catalyzed by the wild-type, K39A, and Y265A mutant alanine racemases.

	(4' <i>R</i> )-[4- <sup>3</sup> H]PMP <sup>a</sup>		(4' <i>S</i> )-[4- <sup>3</sup> H]PMP <sup>b</sup>	
	<sup>3</sup> H released dpm	(%)	<sup>3</sup> H released- dpm	(%)
apo-wild-type	335	52	299	33
apo-K39A	33	5	0	0
apo-Y265A	357	56	50	5
without enzyme	0	0	0	0
apo-AspAT <sup>c</sup>	84	13	595	65
apo-BCAT <sup>c</sup>	682 <sup>c</sup>	68	82	9

<sup>a</sup> The initial radio activity in the reaction mixture with the wild-type and mutant alanine racemases were 639 dpm. That with aminotransferases were 996 dpm.

<sup>b</sup> The initial radio activity in the reaction mixture was 917 dpm.

<sup>c</sup> Reactions with AspAT and BCAT were carried out for estimation of the stereospecificity for the label of the prepared PMPs.

AspAT and BCAT stereospecifically abstract (4'*S*)- and (4'*R*)-hydrogen from PMP under the conditions, respectively (40).

## DISCUSSION

Two mechanisms have been proposed for enzymatic racemization: a “two-base mechanism” and a “one-base mechanism” (48, 49). In the two-base mechanism, two different bases participate in the catalysis; one abstracts the hydrogen from a substrate and the other returns a hydrogen to the deprotonated intermediate. Cofactor-independent racemases, such as glutamate racemase (50, 51), aspartate racemase (52), proline racemase (48, 53), and diaminopimelate epimerase (54), are considered to catalyze racemization by this mechanism. In the one-base mechanism, a single amino acid residue abstracts the  $\alpha$ -hydrogen from a substrate and non-stereospecifically returns it to the anionic intermediate. If the reaction proceeds through the single base mechanism, one can expect that the  $\alpha$ -hydrogen derived from the substrate will be retained at the  $\alpha$ -position of the product (48). Such an internal retention of the  $\alpha$ -hydrogen was observed in the reactions catalyzed by two PLP-dependent racemases: amino acid racemase with low substrate specificity from *Pseudomonas striata* (55) and  $\alpha$ -amino- $\epsilon$ -caprolactam racemase from *Achromobacter obae* (56). Thus, it had been supposed that PLP-dependent racemases including alanine racemase proceed through a single-base mechanism (55-57). However, Floss and his colleague



proposed two base mechanism for the *Ps. striata* amino acid racemase on the basis of the complete disagreement between the substrate enantiomers examined for the relative rates of deuterium incorporation from  $^2\text{H}_2\text{O}$  into separate enantiomers (58). Sawada *et al.* has presented kinetic evidence to show that the alanine racemase reaction follows a two-base mechanism (4). On the basis of stereochemical analysis of the hydrogen abstraction from PMP, Lim *et al.* have suggested that different catalytic residues are situated on each side of the cofactor in the active-site of amino acid racemase with low substrate specificity (47). A series of x-ray crystallographic studies of alanine racemase of *B. stearothermophilus* (6-8) have suggested that the reaction proceeds through a two-base mechanism with Lys39 and Tyr265' as catalytic residues. The active-site structure of the enzyme bound with (*R*)-1-aminoethylphosphonic acid (L-alanine phosphate: L-Ala-P), a synthetic L-alanine analogue, showed that Tyr265' is 3.2 Å from the  $\alpha$ -carbon, in position to act as a general base (7). Sun and Toney sustained the speculation that Tyr265' serves as a catalytic base through the site-directed mutagenesis study of Arg219 interacting with pyridine nitrogen of PLP (5). Mutation of Arg219 increased primary isotope effects but unchanged solvent isotope effect in the L  $\rightarrow$  D direction and increased solvent isotope effect but unchanged primary isotope effect in the D  $\rightarrow$  L direction. Arg219 and Tyr265' are connected through His166

via hydrogen bonds. Thus, they concluded that the observed results are derived from the perturbation of the catalytic residue, Tyr265', mediating the  $\alpha$ -hydrogen abstraction of L-alanine and hydrogen transfer from water to the  $\alpha$ -position of the deprotonated intermediate to form D-alanine.

I have also embarked on the research to verify the two-base mechanism of alanine racemase. I have studied the role of Lys39 of alanine racemase of *B. stearothermophilus* by replacing it site-specifically with alanine (37). The mutant enzyme turned out to be inherently inactive, but gained an activity as high as about 0.1% of that of the wild-type enzyme upon addition of 0.2 M methylamine. The amine-assisted activity of the mutant enzyme depended on the  $\text{pK}_a$  values and molecular volumes of the alkylamines used. A strong kinetic isotope effect was observed when  $\alpha$ -deuterated D-alanine was used as a substrate in the methylamine-assisted reaction, but little effect was observed using its antipode. In contrast, only L-enantiomer of alanine showed a solvent isotope effect in deuterium oxide in the methylamine-assisted reaction. Thus, I conclude that methylamine serves as a base not only to abstract the  $\alpha$ -hydrogen from D-alanine but also to transfer a proton from water to the  $\alpha$ -position of the deprotonated (achiral) intermediate to form D-alanine. The exogenous amine can be regarded as a functional group fully representing Lys39 of the wild-type enzyme. Lys39 of the wild-type

enzyme probably acts as the base catalyst specific to the D-enantiomer of alanine.

My present results demonstrate that conversion of Tyr265' to alanyl residue makes the transamination reactions catalyzed by alanine racemase stereospecific. Alanine racemase catalyzing the transamination from both D- and L-alanine to PLP specifically lost the activity with L-alanine by the mutation. The mutation also abolished the activity of the pro-*S* hydrogen abstraction from the C-4' of PMP occurring during the transamination from PMP to pyruvate but retained the activity of the pro-*R* hydrogen abstraction. These results suggests that Tyr265' is a catalytic residue for the transfer of hydrogen between C-2 of L-alanine and C-4' of the cofactor. The catalytic residue abstracting the hydrogen from C-2 of substrates in the transamination most probably catalyze the same reaction in the racemization.

Sun and Toney described the importance of hydrogen-bond network formed by Arg219, His166, and Tyr265' in lowering the pK<sub>a</sub> of Tyr265', which would be required for Tyr265' to be an effective base catalyst. However, our results showed that the mutation of His166 to alanyl residue retained about 15% activity of the wild-type enzyme in both directions of racemization. Further studies such as x-ray crystallography and pH-profiles of the H166A mutant enzyme

are required to figure out the hydrogen-bond network.

## SUMMARY

I have shown that lysine39 (Lys39) anchoring the cofactor, pyridoxal 5'-phosphate, of the *Bacillus stearothermophilus* alanine racemase acts as the base catalyst specific to the D-enantiomer of alanine through the site-directed mutagenesis studies of Lys39 (Watanabe, A., Kurokawa, Y., Yoshimura, T., Kurihara, T., Soda, K., and Esaki, N. (1999) *J. Biol. Chem.* **274**, 4189-4194). The data also showed that another residue specific to the L-enantiomer in the wild-type enzyme is kept intact in the K39A mutant enzyme. X-ray crystallography of the enzyme suggests that tyrosine265' (Tyr265') serves as the catalytic residue specific to L-enantiomer of substrate amino acid by removing (or returning)  $\alpha$ -hydrogen from (or to) the L-isomer (Stamper, G. F., Morollo, A. A., and Ringe, D. (1998) *Biochemistry* **37**, 10438-10443). In this work, I have studied the role of Tyr265' by site-directed mutagenesis. Mutation of Tyr265' to alanyl, seryl, and phenylalanyl residues virtually inactivated the enzyme. The wild-type enzyme catalyzes the transamination as a side-reaction with both D- and L-alanine to form pyridoxamine 5'-phosphate (PMP). In contrast, Tyr265'→Ala (Y265A) mutant enzyme catalyzed the transamination only with D-alanine: L-alanine

was inert as a substrate. When the apo-wild-type enzyme reacted with (4'*S*)- and (4'*R*)-[4'-<sup>3</sup>H]PMP in the presence of pyruvate, tritium was released from both enantiomers of PMP. However, Y265A mutant enzyme catalyzed the tritium release stereospecifically from the *R*- isomer. These results indicate that Tyr265' is a catalytic residue specific to L-alanine as predicted by x-ray crystallography.

## Chapter 4

### **X-Ray Crystallographic Studies of Alanine Racemase Bound with *N*-(5'-Phosphopyridoxyl)-L- or -D-Alanine**

#### **INTRODUCTION**

Three-dimensional structure of the PLP form of alanine racemase of *Bacillus stearothermophilus* (BSAR) was first reported by Shaw *et al.* (6) with 1.9 Å resolution. The enzyme is composed of two identical subunits with the molecular mass of 43.3 kDa. The subunit is divided into two domains: N-terminal domain consists of  $\alpha/\beta$ -barrel and C-terminal domain consists of  $\beta$ -barrel. Each subunit has one PLP molecule which is bound with  $\epsilon$ -amino group of Lys39 through the internal Schiff base. On the basis of the active-site structure, Shaw *et al.* have suggested that Lys39 and Tyr265' are catalytic bases. In 1998, Stamper *et al.* (7) have reported the structure of BSAR bound with (*R*)-1-aminoethylphosphonic acid (L-Ala-P), a synthetic L-alanine analogue (59). L-Ala-P forms an external Schiff base with PLP in the active-site of BSAR. Because the formed external aldimine is neither racemized nor efficiently hydrolyzed and has a remarkably long half-life, estimated 25 days, the



reaction of L-Ala-P with BSAR results in the inactivation of the enzyme. Stamper *et al.* has suggested that inferred position of  $\alpha$ -hydrogen of the bound L-Ala-P is located within reach of the hydroxyl group of Tyr265'. Recently, Morollo *et al.* have reported the structure of the alanine racemase bound with propionate which is considered to be a Michaelis complex analogue (8). The carboxyl group of propionic acid which probably corresponded to that of the substrate amino acid is found to interact with amide N of Met 312', Arg136, and Tyr265'.

In the previous chapters, I have suggested that alanine racemase reaction proceeds through the two base mechanism with Lys39 and Tyr265' as catalytic residues. Lys39 and Tyr265' serve as the catalytic residue specific to D- and L-alanine, respectively, by removing (or returning)  $\alpha$ -hydrogen from (or to) each isomer. I attempted to confirm the roles of the two residues on the basis of three-dimensional structure of the enzyme bound with the analogues of reaction intermediate. In this work, I have studied the three-dimensional structure of the enzyme bound with *N*-(5'-phosphopyridoxyl)-L- or D-alanine (PLP-L-Ala or PLP-D-Ala, respectively) (Fig. 4-1). Both are the analogues of the intermediates of the racemase reaction, the external aldimines formed from PLP and substrates. These analogues are synthesized by reduction of the

aldimine double bond between PLP and L- or D-alanine, and used as a specific ligand of PLP enzymes such as AspAT and D-AAT in the x-ray crystallographic studies (60-63). Since the alanine moieties of PLP-L-Ala and PLP-D-Ala show the same chiralities as those of L- and D-alanine, crystal structure of the enzyme bound with each analogue probably reflects that of the external aldimine intermediate.

In this chapter, I describe the crystal structures of alanine racemases bound with PLP-L-Ala or PLP-D-Ala at 2.0 Å resolution. I show the structural evidences that alanine racemase reaction proceeds through two base mechanism with Lys39 acting as a base catalyst specific to D-alanine and Tyr265' specific to L-alanine.

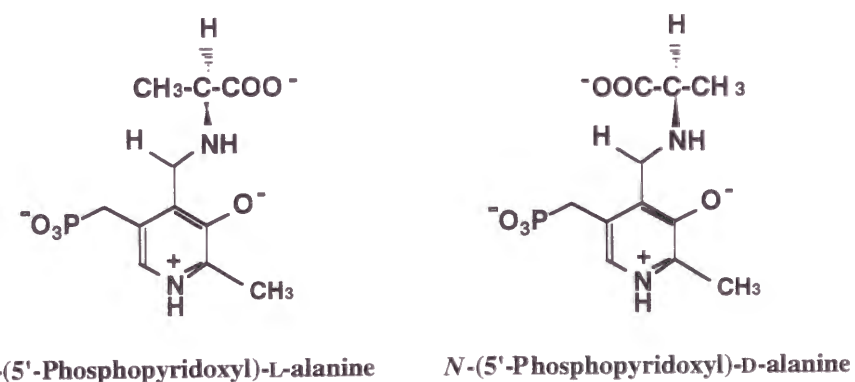


Fig. 4-1. Structures of the external aldimine intermediate analogues.

## EXPERIMENTAL PROCEDURES

### Materials

Alanine racemases was prepared as described in Chapter 2. Apo form of the enzyme was obtained according to the previous methods (13). Dowex 1-X8 was from Dow Chemicals (U.S.A.). Crystal Screen<sup>TM</sup> was from Hampton Research (Riverside, CA, U.S.A.). All other reagents and chemicals were of analytical grade.

### Assays

Protein concentrations were determined as described in Chapter 2. The enzymatic activity of racemization was determined as described in Chapter 2.

### Spectrophotometric Measurements

Absorption spectra were taken with a Shimadzu MPS-2000 or Beckman DU-640 spectrophotometers.

### Synthesis of *N*-(5'-phosphopyridoxyl)-D- or L-Alanine

PLP-L- and PLP-D-Ala were synthesized by the reported methods (63, 64). The reaction mixture containing 2 mmol of PLP and 10 mmol of L-alanine or D-alanine was adjust to pH at 9.3 with

KOH, and filled up to 20 ml with H<sub>2</sub>O. The solution was stirred for 60 min at room temperature, followed by the addition of 5.2 mmol of NaBH<sub>4</sub> for the reduction of the Schiff base formed between PLP and D- or L- alanine. After the yellow color of the solution vanished, equal volume of 0.2 M formic acid was added to the reaction mixture. Then the mixture was applied to a Dowex 1 x 8 column (ϕ3.0 x 15 cm) which had been equilibrated with 0.1 M formic acid. PLP-L- and PLP-D-Ala were eluted with a linear gradient from 0.1 to 1.0 M formic acid. Fractions containing the product were confirmed by thin layer chromatography (TLC) with cellulose plate as a layer, n-butanol saturated 1 N HCl as a solvent, and Gibbs' reagent (*N*-2,6-trichloro-*p*-benzoquinone imine in 1% ethanol) as a detection reagent. A 5 µl of each fraction was spotted on cellulose plate and separated for about 60 min in chromatographic chamber. Then the plate was sprayed with Gibbs' reagent and exposed to ammonia vapor till the blue color appeared. The fractions containing the products were combined and evaporated to dryness with a vacuum rotary evaporator. Formation of PLP-L- and PLP-D-Ala were confirmed with <sup>1</sup>H-NMR. The chemical shifts of each analogue were at 8.1 ppm (s, 1H), 5.0 ppm (d, 2H), 4.5 ppm (s, 2H), 3.9 ppm (q, 1H), 2.6 ppm (s, 3H), and 1.5 ppm (d, 3H).

## Crystallization

Crystals of BSAR bound with PLP-L- or PLP-D-alanine were obtained by crystallization of the complex of apo-wild-type enzyme and PLP-L- or PLP-D-Ala. The reaction mixture (1 ml) containing 23 nmols of apo-wild-type enzyme, each 200 nmols of PLP-L- or PLP-D-Ala and 100 nmols of Tris-HCl buffer (pH 8.5) was incubated for 6 hours at room temperature and dialysed overnight against 100 mM Tris-HCl buffer (pH 8.5). The dialyzed enzyme solutions were concentrated (9 - 10 mg/ml) with Centricon 30 concentrator. The crystals were obtained by hanging-drop vapor diffusion method. The hanging-drop solution was prepared by mixing 5  $\mu$ l of protein solution at 10 mg/ml and 5  $\mu$ l of a reservoir solution consisting of 20% (w/v) poly(ethylene glycol) (PEG) #4000, 200 mM sodium acetate, and 100 mM Tris-HCl buffer (pH 8.5). Drops were equilibrated against a reservoir solution at 20 °C for about 1 week, and the colorless crystals were obtained.

## Data Collection

Both crystals of BSAR bound with PLP-L- and PLP-D-Ala belonged to the space group *P*2<sub>1</sub>2<sub>1</sub>2<sub>1</sub> (Crystal system is orthorhombic). Unit cell dimensions of BSAR bound with PLP-L-Ala were *a* = 99.96 Å, *b* = 90.87 Å, *c* = 85.42 Å,  $\alpha$ ,  $\beta$ , and  $\gamma$  = 90°, and those of that bound with PLP-D-Ala were *a* = 99.79 Å, *b* = 90.72 Å, *c*

= 85.27 Å,  $\alpha$ ,  $\beta$ , and  $\gamma$  = 90°. The asymmetric unit of each crystal contained two subunit molecules. Diffraction data were collected with a Siemens Hi-star detector using CuK $\alpha$  radiation (= 1.5418 Å) produced by a rotating anode generator at 45 kv and 90 mA (Mac Science M18XHF). Distance between crystal and detector was 15 cm. For the analyses of the enzymes combined with PLP-L- and PLP-D-Ala, 199,015 and 201,757 reflections were collected to 1.93, and 1.94 Å resolution, respectively. The data were processed, merged, and scaled with the program of SAINT (Siemens Analytical x-ray Instruments, Inc., Madison, WI, USA). Those data collection and refinement statistics were shown in Table 4-1.



Table 4-1. Data collection and refinement statistics for BSAR bound with PLP-L-Ala or PLP-D-Ala.

	BSAR with PLP-L-Ala	BSAR with PLP-D-Ala
Space group	<i>P</i> 212121	<i>P</i> 212121
Cell dimensions (Å)		
a, b, c	99.96, 90.87, 85.42	99.79, 90.72, 85.27
Subunits / asymmetric unit	2	2
Data collection		
Measured reflections	199,015	201,757
Unique reflections	52,427	40,473
<i>R</i> -sym (%)	8.8	7.7
Resolution (Å)	1.93	1.94
Completeness (%)	88.4	86.9
Refinement		
Resolution (Å)	7.0-2.0	8.0-2.0
Used for refinement	41,051(79.2%)	39,514(75.9%)
Residues/waters	766/313	766/265
Average B-factor(Å <sup>2</sup> )	20.58	20.04
RMS deviation		
bond (Å)	0.007	0.007
angle (deg)	1.33	1.33
<i>R</i> -factor (%)	18.1	18.0
Free- <i>R</i> (%)	24.2	23.3

## Structure Determination and Refinement

All refinement calculations were carried out with X-PLOR 3.851 program (65) and manipulation of the atomic model was carried with TURBO-FRODO program (Biograph). I determined the structure of BSAR bound with PLP-L-Ala first, then determined that of that bound with PLP-D-Ala on the basis of the structure of the former complex. For the construction of the model with PLP-L-Ala, I referred the structure of PLP form of alanine racemase (Brookhaven Protein Data Bank code 1SFT,(6)) as a starting model to run the rigid-body refinement program. Further refinement of the model was carried out with positional and individual temperature refinement (B-factor refinement) programs of X-PLOR 3.851 (65). The regions with conformational changes were checked on the basis of the  $2F_{\text{obs}} - F_{\text{calc}}$  (contoured at  $1\sigma$ ) and  $F_{\text{obs}} - F_{\text{calc}}$  (contoured at  $3\sigma$ ) difference electron density map, and atomic model was moved to fit a electron density map with the program TURBO-FRODO. The cycle of positional refinement, B-factor refinement, and manipulation of atoms were repeated until the crystallographic *R*-factor decreased below 22%. After that, water and analogue molecules were added to the model, followed by several cycles of positional and B-factor refinements. Models of PLP-L- and PLP-D-Ala were constructed by the program X-PRO2D.

## RESULTS AND DISCUSSION

### Quality of Refined Structure

I have determined the structure of alanine racemase bound with PLP-L-Ala to 1.93 Å resolution with the crystallographic *R*- and *R*-free factors of 18.1 and 24.2%, respectively, with using 52,427 reflections at 7.0 - 2.0 Å. The rms deviations of bond length, bond angles, dihedral angles, improper angles are 0.007 Å, 1.33°, 26.2°, and 0.69°, respectively. The final model contains 766 amino acid residues, 313 water molecules, and 2 PLP-L-Ala molecules. A Ramachandran plot (66) of the model is shown in Fig. 4-2 (A). Analysis of protein using PROCHECK program (67) has shown that 88.9% of all the residues are in the most favoured regions of a Ramachandran plot, 10.5% of the residues are in additional allowed regions, and 0.6% of residues are in generously allowed regions. The maximum coordinate error of 0.215 Å was estimated from a Luzzati plot (68).

On the other hand, I have determined the structure of alanine racemase bound with PLP-D-Ala to 1.94 Å resolution with the crystallographic *R*- and *R*-free factors of 18.0 and 23.3%, respectively, on the basis of 50,473 reflections at 8.0 - 2.0 Å. The rms deviations of bond length, bond angles, dihedral angles, improper

angles are 0.007 Å, 1.33°, 26.53°, and 1.98°, respectively. The final model contains 766 amino acid residues, 265 water molecules, and 2 PLP-D-Ala molecules. A Ramachandran plot (66) of the final model is shown in Fig. 4-2 (B). Analysis of the protein using PROCHECK program (67) has shown that 91.1% of all the residues are in the most favoured regions, 8.0% of the residues are in additional allowed regions, and 0.9% of residues are in generously allowed regions. The maximum coordinate error was calculated to 0.212 Å from a Luzzati plot (68).

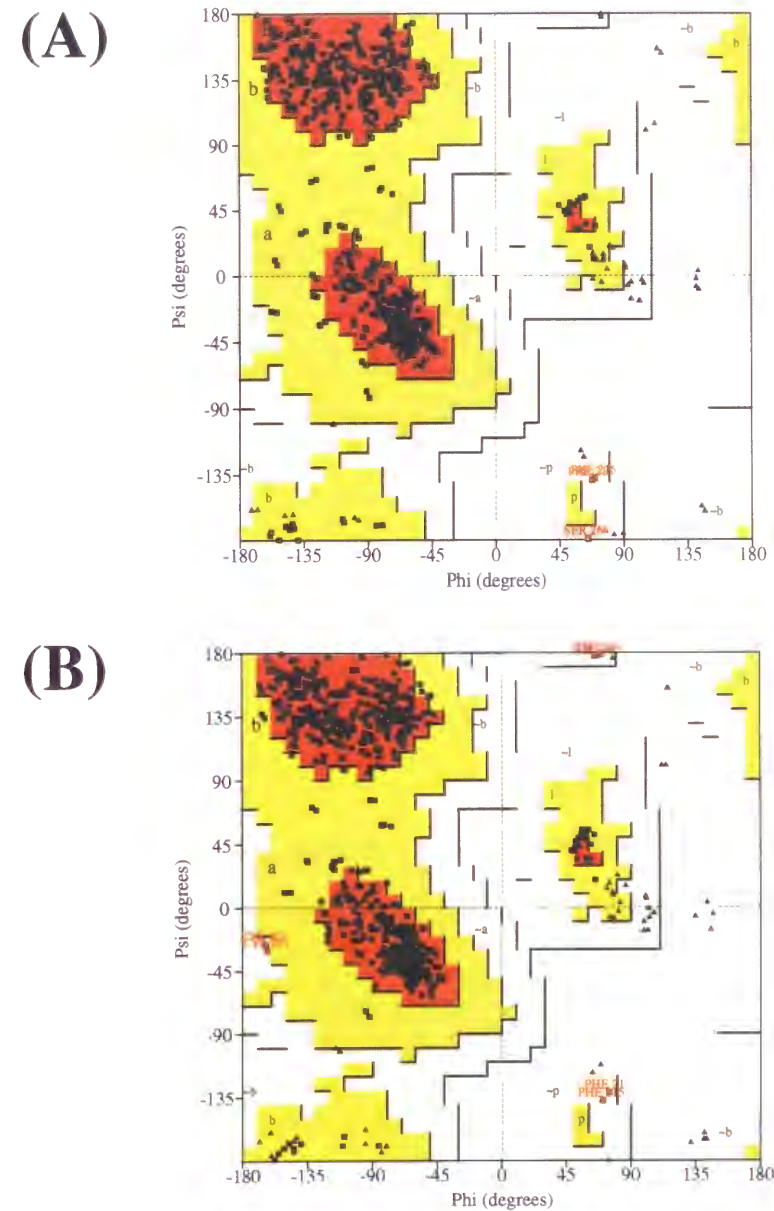


Fig. 4-2. Ramachandran plot of the backbone torsion angles of BSAR bound with PLP-L-Ala (A) and that with PLP-D-Ala (B). Triangles and squares indicate glycine and non-glycine residues, respectively.



### **Comparison of Crystal Structures between PLP-form BSAR and BSAR bound with PLP-L- or PLP-D-Ala**

I have compared the refined structures of BSAR bound with PLP-L- or D-Ala to that of the PLP form of the enzyme (6). The rms deviations of C $\alpha$  between the PLP-form and PLP-L-Ala-bound structures are 0.24 Å (A chain) and 0.28 Å (B chain). On the other hand, the rms deviations of C $\alpha$  between the PLP-form and PLP-D-Ala-bound structures are 0.24 Å (A chain) and 0.22 Å (B chain). That values indicate that the gross structures of BSAR bound with PLP-L- or PLP-D-Ala are similar to that of the PLP-form BSAR. Stamper *et al.* (7) also reported no major conformation change between the PLP-form and L-Ala-P-bound structure. The main-chain conformation of BSAR probably was not significantly changed by binding of substrates and by the formation of external aldimine.

### **Substrate Binding Site**

Shaw *et al.* (6) found that one acetate molecule was bound to the active-site of the PLP-form BSAR. Acetate molecule behaved as a competitive inhibitor of BSAR with  $K_i$  value of  $92 \pm 14$  mM (with sodium acetate) (8). Formation of two hydrogen bonds were suggested on the basis of the crystal structure between the carboxyl group of acetate and the main chain nitrogen of Met312' from the

other subunit, and the NH1 of Arg136. Morollo *et al.* has determined the structure of the enzyme containing propionate which serves as an inhibitor with  $K_i$  value of  $20 \pm 3$  mM (8). They have suggested that the hydrogen bonds also forms between the carboxyl group of propionate and the backbone amide of Met312', and the guanidino group of Arg136. In the structures of BSAR bound with PLP-L- or PLP-D-Ala, similar two hydrogen bonds are observed between the carboxyl groups of both L- and D-alanine moiety and the backbone amide of Met312' and the guanidino group of Arg136. The distances from one carboxylate oxygen to main-chain nitrogen of Met312' and the other carboxylate oxygen to NE1 of Arg136 are approximately 2.8 Å and 3.0 Å, respectively, in both the structures. Methyl groups of both L- and D-alanine moiety in both structures probably interact with Met312' and Tyr354 through the van der Waals contacts (C-C distance < 4.5 Å).

No difference in the mode of binding between L-alanine and D-alanine moieties of the external aldimine analogues suggests that carboxyl group and methyl group of both enantiomers of substrate are bound to the same position of the enzyme as depicted in Fig. 4-3.

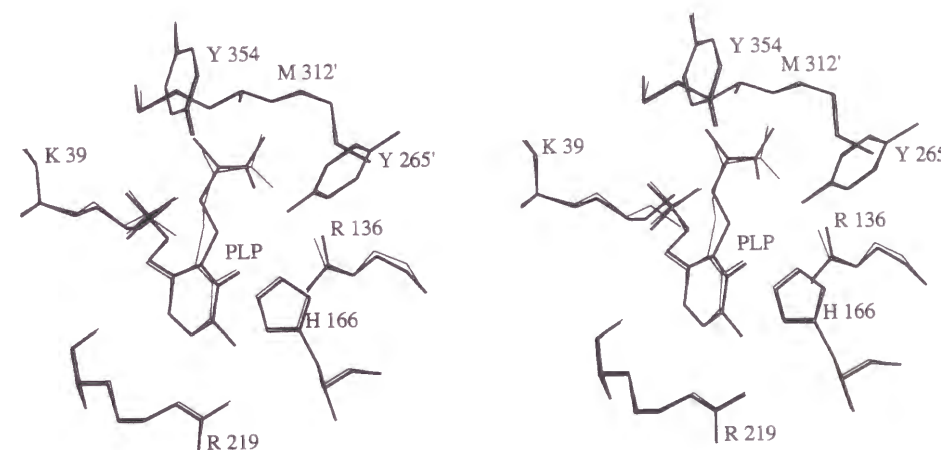


Fig. 4-3. Stereodiagram of the active site of BSAR bound with PLP-L-Ala (thick line) overlaid with that bound with PLP-D-Ala (thin line).

## Catalytic Residues

The main-chain ( $C\alpha$ ) positions of PLP-form BSAR are nearly the same as those of the enzymes bound with PLP-L- or PLP-D-Ala. However, the active-site structure of BSAR is changed by the insertion of these external aldimine analogues. In the PLP form of BSAR, PLP is bound with the NZ of Lys39 through the internal Schiff base. The refined structures of the enzymes bound with PLP-L- or PLP-D-Ala tilt outward from the protein approximately  $24^\circ$  relative to the internal aldimine as shown in Fig. 4-4 and 4-6. Both analogues take the middle position between the proposed catalytic residues, Lys39 and Tyr265'. The distances between these residues and  $C\alpha$  of both L- and D-alanine moieties of the external aldimine analogues are approximately 3.5 Å. The distance allows both residues to serve as catalytic residues abstracting  $\alpha$ -hydrogen from substrate and adding hydrogen to  $\alpha$ -position of substrate.

Structure of external aldimine is considered to be planar, because the amino group of substrate is covalently bound with  $C4'$  of the cofactor with double bond. The planar electron density from N1 of PLP through the imine linkage of the external aldimine derivative in BSAR bound with L-Ala-P (7) is compatible with this structure. However, as shown in Fig. 4-4 and 4-6, PLP-L- and PLP-D-Ala in the active-site of BSAR are not planar, because the C-N linkage between

$C4'$  of the cofactor and amino group (N) of alanine is connected by single bond. Distances between  $C\alpha$  and proposed catalytic residues of BSAR with external aldimine probably differ from those of BSAR bound with its synthetic analogues because of the difference in the angles of  $C4'$ -N-  $C\alpha$  between external aldimine and its analogues. However, the structure of BSAR bound with L-Ala-P have shows that Tyr265' is 3.2 Å from the  $C\alpha$ . The distance is nearly the same of that in the structure of BSAR bound with PLP-L-Ala (3.5 Å). The structure of BSAR bound with PLP-L- and PLP-D-Ala probably mimic that of the enzyme with external aldimine fairly well.

I have suggested that Lys39 and Tyr265' serve as the catalytic residue specific to D- and L-enantiomer of substrate amino acid by removing (or returning)  $\alpha$ -hydrogen from (or to) each isomer, respectively. Sun and Toney also showed the evidence that Tyr265' acts as a catalytic base specific to L-alanine through the site-directed mutagenesis studies of Arg219. On the basis of the structure of BSAR bound with PLP-L-Ala, the  $\alpha$ -H of L-alanine moiety is deduced to be located near the phenolic oxygen of Tyr265'. In contrast,  $\alpha$ -H of D-alanine moiety of PLP-D-Ala is deduced to be located near the  $\epsilon$ -amino group of Lys39 (Fig. 4-5 and 4-7). Furthermore, the distances between phenolic oxygen of Tyr265' and CA, N, and  $C4'$  of PLP-L-Ala are 3.5 Å, 3.8 Å, and 3.1 Å, respectively (Fig. 4-8). In the structure of BSAR bound with PLP-D-Ala, the distances between  $\epsilon$ -



amino group of Lys39 and CA, N, and C4' of PLP-D-Ala are 3.5 Å, 2.5 Å, and 3.4 Å, respectively, (Fig. 4-9). These results confirms the speculation that Lys39 and Tyr265' are catalytic residues being responsible for the proton abstraction from D- and L-alanine, respectively.

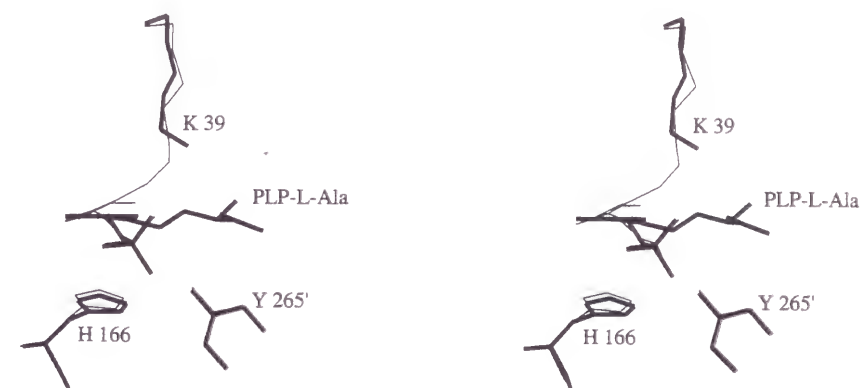


Fig. 4-4. Stereodigram of the active-site of PLP-form BSAR (thin line) overlaid with BSAR bound with PLP-L-Ala (thick line).

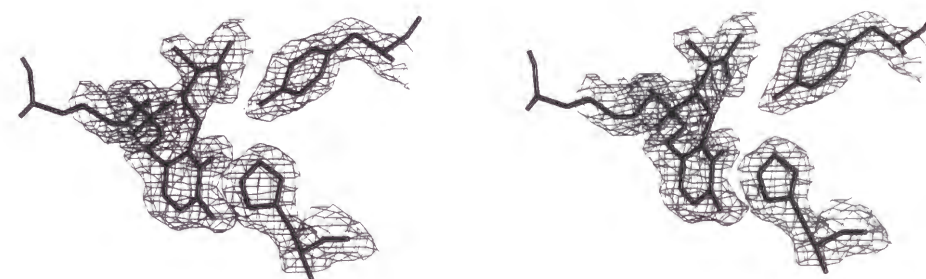


Fig. 4-5. Stereoview of BSAR bound with PLP-L-Ala. The electron density shown was calculated using a simulated annealing omit map with coefficients  $2F_o - F_c$  map contoured at 1.0 sigma.

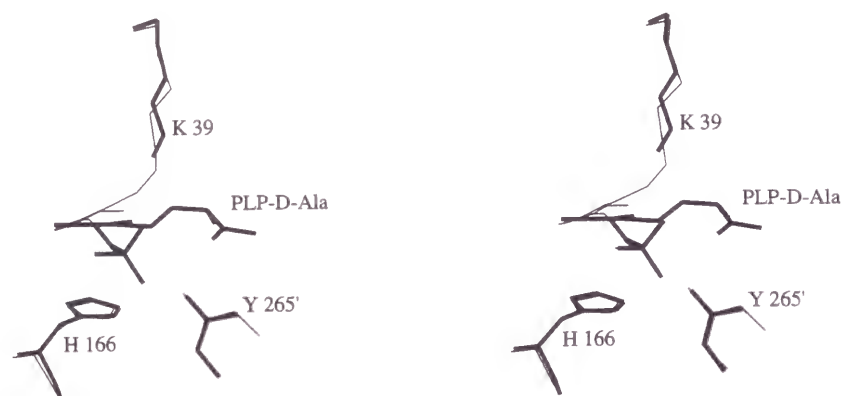


Fig. 4-6. Stereodiagram of the active-site of PLP-form BSAR (thin line) overlaid with BSAR bound with PLP-D-Ala (thick line).

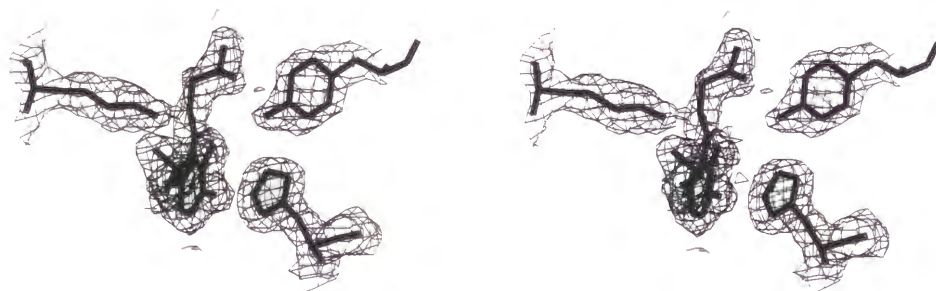


Fig. 4-7. Stereoview of BSAR bound with PLP-D-Ala. The electron density shown was calculated using a simulated annealing omit map with coefficients  $2Fo-Fc$  map contoured at 1.0 sigma.

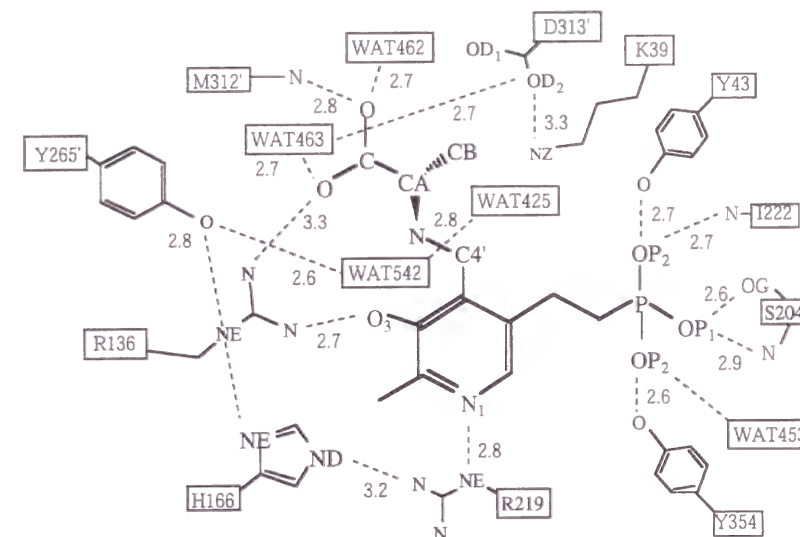


Fig. 4-8. Schematic diagram of the active-site of BSAR bound with PLP-L-Ala. Presumed hydrogen bonds are indicated by dashed lines between atoms that are separated by less than 3.3 Å.

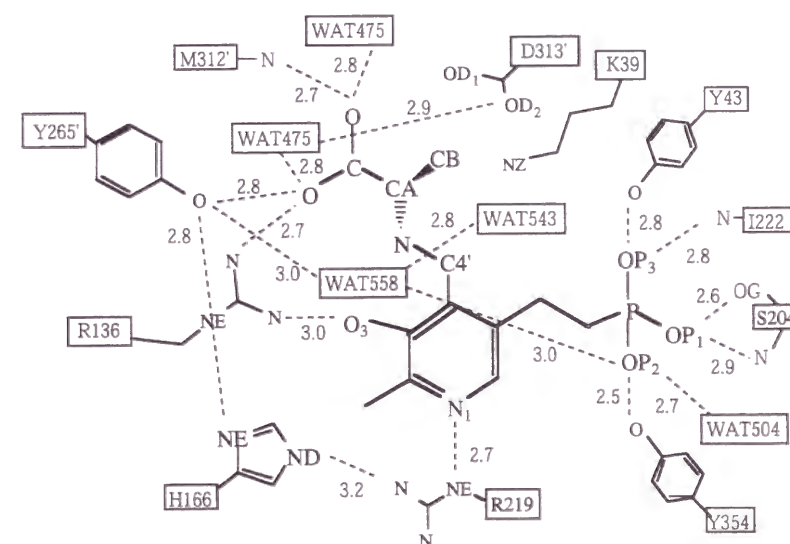


Fig. 4-9. Schematic diagram of the active-site of BSAR bound with PLP-D-Ala. Presumed hydrogen bonds are indicated by dashed lines between atoms that are separated by less than 3.3 Å.

## SUMMARY

The crystal structures of alanine racemases bound with *N*-(5'-phosphopyridoxyl)-D- or L-alanine (PLP-L-Ala or PLP-D-Ala, respectively) were determined at both 2.0 Å resolution with crystallographic *R* factor of 18.3 and 18.0, respectively. Three-dimensional structure of these complexes show that both analogues take the middle position between the proposed catalytic residues, Lys39 and Tyr265'. Methyl and carboxyl groups of the alanine moieties of both PLP-L- and PLP-D-Ala seem to occupy the similar position in the active-site and to interact with the same residues of the enzyme. On the basis of the configuration of the alanine moieties of PLP-L- and PLP-D-Ala,  $\alpha$ -H of L- and D-enantiomers are inferred to be located near the phenolic oxygen of Tyr265' and located near the  $\epsilon$ -amino group of Lys39, respectively.

In consequence, I conclude that racemization of alanine proceeds through two base mechanism with Lys39 acting as a base catalyst specific to D-alanine and Tyr265' specific to L-alanine.

## Chapter 5

### Role of Tyrosine265' in $\alpha,\beta$ -Elimination of $\beta$ -Chloroalanine

#### INTRODUCTION

Alanine racemase [EC 5.1.1.1] is a pyridoxal 5'-phosphate (PLP) enzyme that occurs widely in bacteria and plays a central role in the metabolism of D-alanine, an essential component of peptidoglycans in bacterial cell walls (69). As described in Chapter 2, we constructed a mutant enzyme of alanine racemase from *B. stearothermophilus* (BSAR) by substitution of an alanyl residue for lysine39 (Lys39) binding with PLP (13). Through chemical rescue studies with Lys39→Ala (K39A) mutant enzyme, I showed that Lys39 bears two major roles as follows. Lys39 serves as a catalytic base specifically directing to D-enantiomer of substrate amino acid: it abstracts  $\alpha$ -hydrogen from D-alanine and transfers a proton from water to the  $\alpha$ -position of the deprotonated (achiral) intermediate to form D-alanine (Fig. 5-1; steps a and b). Another important role of Lys39 is to mediate transaldimination: it facilitates formation of PLP-aldimine of a substrate amino acid (Fig. 5-1; steps 1 and 2) (37). X-ray



crystallographic studies of the wild-type enzyme suggested that tyrosine265' (Tyr265') is the counterpart residue of Lys39 mediating specifically  $\alpha$ -hydrogen transfer of L-enantiomer of substrate amino acid (Fig. 5-1; steps c and d) (6-8). As described in previous chapters, I have confirmed the role of Tyr265' through the studies of Tyr265'  $\rightarrow$  Ala (Y265A) mutant enzyme and crystallography of BSAR bound with PLP-L- or D-Ala.

In this chapter, I describe the role of Lys39 and Tyr265' in the  $\alpha,\beta$ -elimination of  $\beta$ -chloroalanine ( $\beta$ -Cl-Ala) which serves as mechanism-based inhibitors of various PLP enzymes. I have found that Tyr265' is not essential for the  $\alpha,\beta$ -elimination of  $\beta$ -Cl-Ala though it is indispensable for alanine racemization.

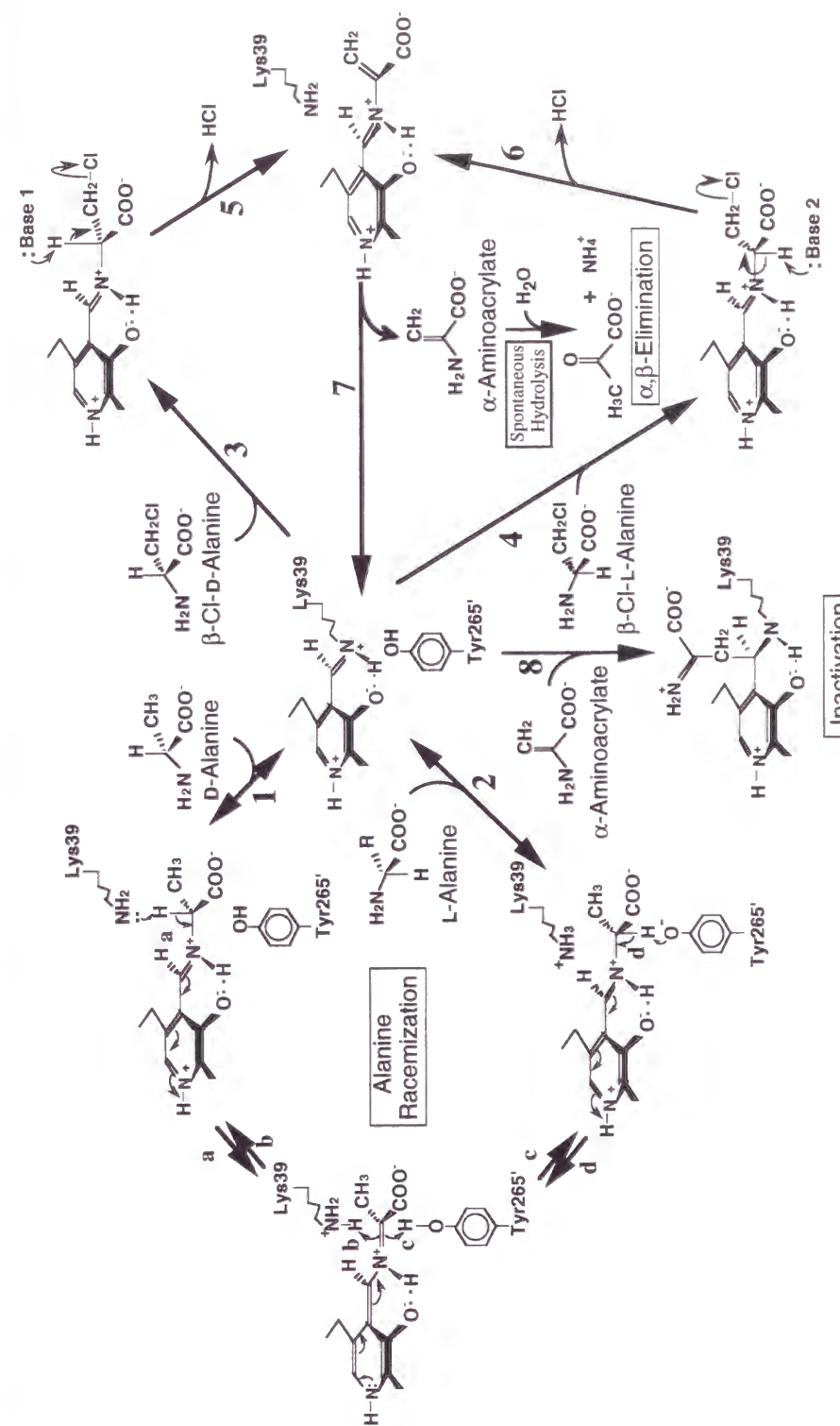


Fig. 5-1. Proposed reaction mechanism of  $\alpha,\beta$ -elimination of  $\beta$ -Cl-Ala catalyzed by alanine racemase.

## EXPERIMENTAL PROCEDURES

### Materials

The wild-type and mutant enzymes of BSAR were overproduced in recombinant *E. coli* JM109 cells and purified by heat-treatment, DEAE-TOYOPEARL 650M, and Butyl-TOYOPEARL 650M column chromatography as described in Chapter 2. Purity of the enzymes were determined by SDS-PAGE.  $\beta$ -chloroalanine ( $\beta$ -Cl-Ala) was purchased from SIGMA. All other reagents and chemicals were of analytical grade.

### Assays

Protein concentrations were determined as described in Chapter 2. The enzymatic activity of racemization was determined as described in Chapter 2. The  $\alpha,\beta$ -elimination of  $\beta$ -Cl-Ala was assayed as follows. The reaction mixture (1 ml) contained 100 mM HEPES buffer (pH 7.2), 0.16 mM NADH, 5.5 units of lactate dehydrogenase, various concentrations of  $\beta$ -Cl-D- or L-Ala. The reaction was started by addition of homogeneous preparations of wild-type and mutant alanine racemases and performed at 37 °C. The decrease in absorbance at 340 nm was monitored with a Shimadzu MPS-2000 spectrophotometer.

## RESULTS AND DISCUSSION

Alanine racemase does not catalyze the racemization of  $\beta$ -Cl-Ala (70) but catalyzes the  $\alpha,\beta$ -elimination of both enantiomers to produce chloride, pyruvate, and ammonium (71, 72).  $\alpha,\beta$ -Elimination of  $\beta$ -Cl-Ala differs markedly from racemization of alanine in that the step of reprotonation at the  $\alpha$ -position of the deprotonated intermediate is not involved. Because Tyr265' acts as a base specifically abstracting  $\alpha$ -hydrogen from L-enantiomer (shown as Base 2 in Fig. 5-1), Y265A mutant enzyme is probably active toward D- $\beta$ -Cl-Ala but inactive toward L- $\beta$ -Cl-Ala. However, as shown in Table 5-1, Y265A mutant enzyme acts on both enantiomers of  $\beta$ -Cl-Ala with similar kinetic parameters as those obtained for the wild-type enzyme. This presents a clear contrast to the activities of the mutant enzyme in the alanine racemization (Table 3-1).

Table 5-1. Kinetic parameters of the wild-type and mutant alanine racemases in  $\alpha,\beta$ -elimination of  $\beta$ -Cl-Ala.

	$\beta$ -Cl-D-Ala			$\beta$ -Cl-L-Ala		
	$k_{\text{cat}}$ ( $\text{sec}^{-1}$ )	$K_{\text{m}}$ (mM)	partition ratio	$k_{\text{cat}}$ ( $\text{sec}^{-1}$ )	$K_{\text{m}}$ (mM)	partition ratio
wild-type	4.6	0.15	72	1.2	2.8	61
Y265A	3.8	0.12	83	1.2	0.54	79
K39A	n.d. <sup>a</sup>	-	-	n.d. <sup>a</sup>	-	-
K39A + MA <sup>b</sup>	0.07	0.030	18	0.07	0.5	419

<sup>a</sup>Activity was detectable neither at pH 7.2 nor 9.0.

<sup>b</sup>The reaction was carried out in the presence of 100 mM methylamine in 100 mM CHES buffer (pH 9.0).

K39A mutant enzyme showed no activity toward both enantiomers of  $\beta$ -Cl-Ala at pH 7.2 or 9.0. This is probably due to inability of the enzyme to catalyze transaldimination (Fig. 5-1; steps 3 and 4). However, exogenous methylamine added to the mutant enzyme facilitated  $\alpha,\beta$ -elimination of  $\beta$ -Cl-Ala in the same manner as observed in alanine racemization (37); methylamine mimics the side chain of Lys39 of the wild-type enzyme and mediates transaldimination to form a PLP aldimine of  $\beta$ -Cl-Ala. Aminoacrylate formed is liberated from the Schiff base by another transaldimination with methylamine through a step similar to step 7 in Fig. 5-1. PLP-binding lysyl residues of aspartate aminotransferase (73), tryptophan synthase (31) D-amino acid aminotransferase (32, 33) and aromatic L-amino acid decarboxylase (34) are known to mediate transaldimination to release reaction products. The methylamine-assisted  $\alpha,\beta$ -elimination was accelerated with increase in pH (Fig. 5-2) and methylamine concentration (Fig. 5-3). These results suggest that deprotonated methylamine is the active species catalyzing the transaldimination reaction. Absorption spectrum of the wild-type enzyme suggests that imine nitrogen of the internal Schiff base is protonated between pH 5.5 - 12.0 (8). This suggests that the transaldimination steps (Fig. 5-1, steps 1, 2, 3, 4 and 7) proceed through the attack on the imine bond by a deprotonated amino group



of substrate or Lys39 in the reactions catalyzed by the wild-type enzyme. The amino groups of substrate amino acid and Lys39 are most probably deprotonated in the alkaline pH region (around 10) corresponding to the optimum pH for the alanine racemization. K39A mutant enzyme showed no activity toward  $\beta$ -Cl-Ala in the absence of methylamine (Fig. 5-3). This suggests that the amino group of  $\beta$ -Cl-Ala does not act as a base substituting for methylamine. This is probably due to steric hindrance by other groups attached to the  $\alpha$ -carbon of  $\beta$ -Cl-Ala.

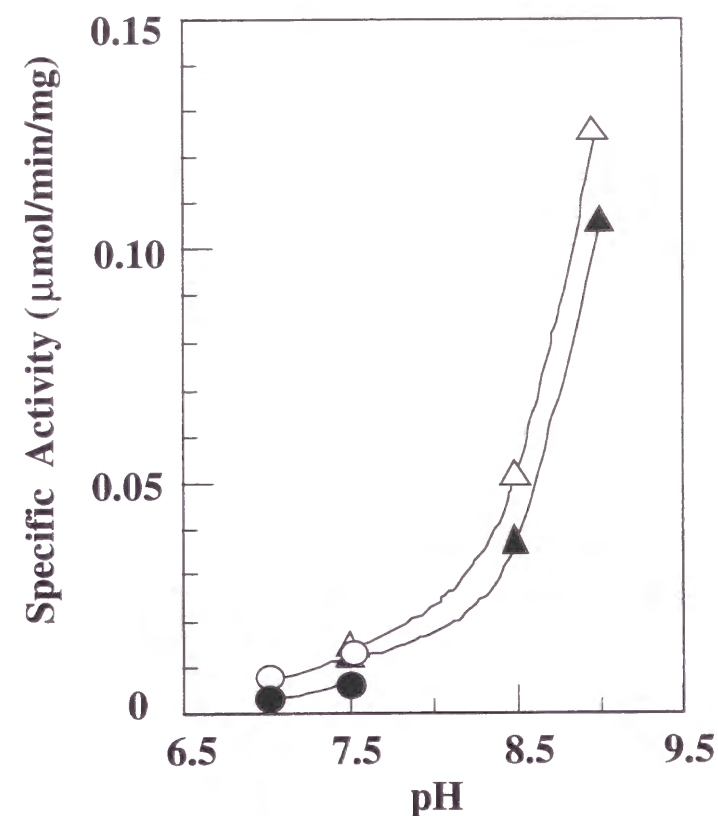


Fig. 5-2. Effect of pH on the rate of  $\alpha,\beta$ -elimination of  $\beta$ -Cl-D- and L-Ala catalyzed by K39A mutant enzyme in the presence of methylamine. The reaction was carried out with 10 mM  $\beta$ -Cl-D-Ala (open symbol) or  $\beta$ -Cl-L-Ala (closed symbol), 100 mM HEPES buffer (circle, pH 7.0 and 7.5) or Bis-Tris-Propane buffer (triangle, pH 7.5, 8.5, and 9.0), and 75  $\mu$ g of a homogeneous preparation of K39A mutant enzyme in the presence of 100 mM methylamine. Other conditions were the same as those described in the EXPERIMENTAL PROCEDURES in this chapter.

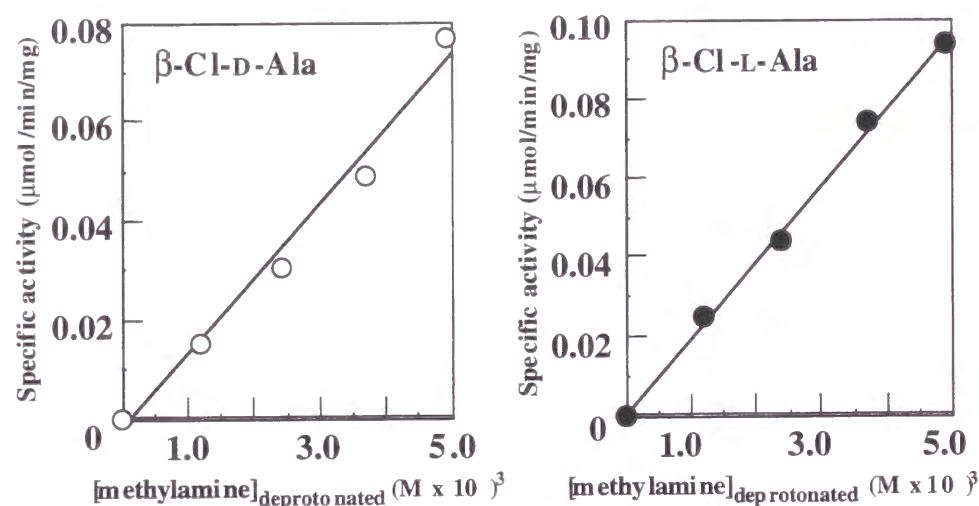


Fig. 5-3. Effect of concentration of methylamine on the rate of  $\alpha,\beta$ -elimination of  $\beta$ -Cl-D- and L-Ala catalyzed by K39A mutant enzyme.

The reaction mixture (1 ml) contained 100 mM CHES buffer whose pH was adjusted to pH 9.0 with tetramethylammonium hydroxide, 0.16 mM NADH, 5.5 units of lactate dehydrogenase, 10 mM  $\beta$ -Cl-D- or L-Ala and various concentrations of methylamine. The ionic strength was maintained at 0.5 with tetramethylammonium chloride. The reaction was started by addition of 65  $\mu$ g of K39A mutant enzyme and performed at 37 °C. The specific activities obtained were plotted against the concentrations of deprotonated methylamine which were calculated from the  $K_a$  value of methylamine (10.6) (12), pH 9.0, and the concentration of methylamine added ( $[\text{methylamine}]_{\text{total}}$ ) according to the equation:

$$[\text{methylamine}]_{\text{deprotonated}} = [\text{methylamine}]_{\text{total}} / (1 + [\text{H}^+]/K_a).$$

D-Amino acid aminotransferase catalyzes  $\alpha,\beta$ -elimination of  $\beta$ -Cl-Ala as a secondary function of the enzyme in the same manner as alanine racemase and other pyridoxal enzymes (74-79). Soper and Manning (79) showed that the step of  $\alpha$ -proton abstraction from the substrate is not rate-limiting in  $\alpha,\beta$ -elimination of  $\beta$ -bromo-D-alanine catalyzed by D-amino acid aminotransferase in contrast to transamination of ordinary D-amino acids catalyzed by the same enzyme. It may be reasonable to assume a mechanism in which  $\alpha$ -hydrogen of  $\beta$ -Cl-Ala is readily liberated without participation of particular basic residues: Bases 1 and 2 in Fig. 5-1 are for example water molecules. The  $\alpha$ -hydrogen removal possibly proceeds concertedly with release of chloride (Fig. 5-1; steps 5 and 6). This mechanism is consistent with our findings that Tyr265' is not required for  $\alpha,\beta$ -elimination of  $\beta$ -Cl-Ala.

Various PLP-enzymes are inactivated by  $\beta$ -Cl-Ala at a rate specific for each enzyme while catalyzing multiple turnover numbers of  $\alpha,\beta$ -elimination. The rate is referred to as a partition ratio and expressed as an average number of  $\alpha,\beta$ -elimination events catalyzed by an enzyme before its complete inactivation. Both the wild-type and Y265A mutant enzymes showed partition ratios similar to each other. This is compatible with the inactivation mechanism of alanine racemases proposed by Walsh and his coworkers (71, 78): PLP-aldimine of the bound lysyl residue is modified with the common achiral product, aminoacrylate, formed in the  $\alpha,\beta$ -elimination (Fig.

5-1; step 8). The K39A mutant enzyme rescued by methylamine showed much lower partition ratios than the wild-type and Y265A mutant enzymes. Methylamine probably serves as a base to accelerate the chemical modification with aminoacrylate, although further studies are required for clarification of the mechanism.

### SUMMARY

Tyr265' of BSAR serves as the catalytic base specific to L-enantiomer of substrate amino acid by removing (or returning)  $\alpha$ -hydrogen from (or to) the isomer. I examined the role of Tyr265' in the  $\alpha,\beta$ -elimination of  $\beta$ -chloroalanine with expectation that Y265A mutant enzyme catalyzes  $\alpha,\beta$ -elimination of only D-enantiomer of  $\beta$ -chloroalanine. However, L- $\beta$ -chloroalanine also served as a substrate; the enantiomer was rather better as a substrate than its antipode. Moreover, the mutant enzyme was equally as active as the wild-type enzyme in the elimination reaction. These indicate that Tyr265' is essential for alanine racemization but not for the  $\alpha,\beta$ -elimination of  $\beta$ -chloroalanine.

## Chapter 6

### General Conclusion

In this work, I have studied the reaction mechanism of thermostable alanine racemase [EC 5.1.1.1] from *Bacillus stearothermophilus* (BSAR) with focusing on the catalytic residues. As described in Chapter 2, the inactive Lys39 $\rightarrow$ Ala (K39A) mutant enzyme shows the activity in the presence of alkylamines. The amine-assisted racemization catalyzed by K39A mutant enzyme was dependent on  $pK_a$  and molecular volumes of added amines. In the methylamine-assisted racemization, a substrate isotope effect was observed only in D- to L-alanine reaction. On the other hand, a solvent isotope effect was observed in L- to D- alanine reaction. On the basis of these results, I have concluded that Lys39, PLP-binding lysyl residue of BSAR, bears two major roles. Lys39 serves as a catalytic base specifically directing to D-enantiomer of substrate amino acid: it abstracts  $\alpha$ -hydrogen from D-alanine and transfers a proton from water to the  $\alpha$ -position of the deprotonated (achiral) intermediate to form D-alanine. Another important role of Lys39 is to mediate transaldimination: it facilitates formation of PLP-aldimine of a substrate amino acid. The results shown in Chapter 2 also suggest



the presence of another catalytic residue which is specific to L-alanine.

A series of studies of x-ray crystallography of BSAR suggest that Tyr265' from the other subunit mediates the abstraction and addition of  $\alpha$ -hydrogen of L-alanine. As described in Chapter 3, I have confirmed the hypothesis through the analyses of the stereospecificity for the transamination catalyzed by Tyr265'→Ala (Y265A) mutant alanine racemase. Transamination catalyzed by BSAR as a side-reaction is characterized by its non-stereospecificity: both D- and L-alanine serve as substrates in the transamination from amino acid to PLP. However, Y265A mutant enzyme catalyzes transamination only with D-alanine: L-alanine is inert as a substrate. The wild-type BSAR catalyzes the tritium abstraction from both (4'S)- and (4'R)-[4'-<sup>3</sup>H]PMP during the transamination from PMP to pyruvate. In contrast, Y265A mutant enzyme abstracts tritium only from (4'R)-[4'-<sup>3</sup>H]PMP specifically. These results suggest that Tyr265' serves as a catalytic residue abstracting  $\alpha$ -hydrogen specifically from L-alanine.

As described in Chapter 4, I have confirmed the roles of Lys39 and Tyr265' by x-crystallography of BSAR bound with *N*-(5'-phosphopyridoxyl)-D- or L-alanine (PLP-L-Ala or PLP-D-Ala, respectively), synthetic analogues of external aldimines. The refined structures of BSAR bound with PLP-L-Ala and PLP-D-Ala suggest that  $\alpha$ -H of alanine moiety of PLP-L-Ala and PLP-D-Ala are located

near the phenolic oxygen of Tyr265' and near the  $\epsilon$ -amino group of Lys39, respectively. On the basis of the results described in Chapter 2-4, I conclude that the alanine racemase reaction proceeds through the two-base mechanism with Lys39 and Tyr265' as catalytic residues being specific to D- and L-alanine, respectively.

Alanine racemase does not catalyze the racemization of  $\beta$ -chloroalanine ( $\beta$ -Cl-Ala) but catalyzes the  $\alpha,\beta$ -elimination of both enantiomers to produce chloride, pyruvate, and ammonium. I have examined the role of Tyr265' in the  $\alpha,\beta$ -elimination of  $\beta$ -Cl-Ala catalyzed by alanine racemase. Y265A mutant enzyme catalyzes  $\alpha,\beta$ -elimination of not only  $\beta$ -Cl-D-Ala but also L-isomer with the comparable rate to that of the  $\alpha,\beta$ -elimination of  $\beta$ -Cl-L-Ala catalyzed by the wild-type enzyme. The results suggested that Tyr265' is essential for alanine racemization but not for the  $\alpha,\beta$ -elimination of  $\beta$ -Cl-Ala.

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